Pharmacology and Clinical Effects of N-acetylcysteine in Neurodegenerative Disorders

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DEDICATION

This thesis is dedicated to my husband, whose support and encouragement kept me going, and to our sons and daughters-in law, who understood my need to pursue its completion.

It is also dedicated to our grandchildren, who somehow understood the need for Nana to work on her school work

Lastly, it is dedicated to the boys with adrenoleukodystrophy and their families, whose courage and strength inspired and motivated me to continue

ABSTRACT

Free radicals and other reactive oxygen species (ROS) constitute a normal part of the intracellular environment. Endogenous enzymes such as catalase, superoxide dismutase and the thiol redox systems, glutathione and oxidized glutathione, serve as reducing agents to minimize the harm ROS might cause in the cell. An excess of ROS, however, can tilt the delicate balance leading to oxidative stress. It is now well known that oxidative stress (OS) plays an important role in many neurodegenerative disorders such as Parkinson disease, Alzheimer's disease, amyotrophic lateral sclerosis and other disorders with neurodegenerative effects, including adrenoleukodystrophy (ALD).

Many antioxidants have been studied in an effort to ameliorate the oxidative stress, slow the progression or treat the symptoms of these and other neurodegenerative disorders, mostly with limited success. The potential reasons for the limited success of these compounds are discussed in this thesis, and the dissertation research on the potential use of N-acetylcysteine, (NAC) a well-known antioxidant and glutathione precursor, is described in detail.

The first portion of the dissertation research described herein focused on characterizing the pharmacokinetics of intravenously administered NAC as adjunctive therapy with hematopoietic cell transplant (HCT) in ALD. The objectives of this research were to characterize the pharmacokinetics of i.v. NAC

and to explore one of the mechanisms by which NAC is thought to exert its antioxidant effects: through the provision of cysteine for the synthesis of glutathione, the most powerful endogenous antioxidant in the body.

The second clinical study described in this thesis focused on the effect of i.v. administered NAC on glutathione concentrations measured directly in the brain of people with Parkinson's disease, Gaucher disease and healthy control subjects, through the use of magnetic resonance spectroscopy.

The research described in this dissertation represents the first report of the pharmacokinetics and direct pharmacodynamic effects of i.v. NAC administration in those affected by disorders of neurodegeneration. This research now serves as the basis for other pharmacokinetic and pharmacodynamic studies of NAC in other populations as well as with other dosage forms and formulations, with the hope of developing effective antioxidant treatments for those suffering from neurodegenerative disorders.

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Chapter 1

Introduction

1.1 Oxidative Stress and the Role of Glutathione in Neurodegenerative Disorders

The metabolism of molecular oxygen results in a variety of molecules and free radicals (chemical species with one unpaired electron) called reactive oxygen species (ROS). Oxidative stress occurs as a consequence of an alteration in the equilibrium of the production of ROS and antioxidative processes, in favor of the production of ROS. In order to avoid damage caused by ROS, such as lipid peroxidation, protein modification, and DNA strand breaks, mechanisms exist which remove ROS or prevent the generation of ROS. Although it is not clear whether oxidative stress is a precursor or a result of disease processes, it is present to some extent in all neurodegenerative disorders.

Compared to other organs, the brain has some disadvantages regarding the generation and detoxification of ROS. The oxygen requirements of the human brain account for 20% of the oxygen consumed by the body, despite the brain being only 2% of body weight[1] The brain is among the major organs generating large amounts of ROS. In cells of the nervous system, the major site of physiological ROS generation is the mitochondria. Approximately 2-4% of the oxygen consumed by mitochondria is diverted to form superoxide(O_2^-) [2] Superoxide dismutase (SOD) converts superoxide to hydrogen peroxide (H_2O_2), which is subsequently converted to water and molecular oxygen by glutathione peroxidase (GPx) or catalase.[3] Hydrogen peroxide can also react with iron via

the Fenton reaction to form hydroxyl radicals, which cause lipid peroxidation.[4] Compared with other organs, the brain is especially vulnerable to oxidative stress because it has lower SOD, catalase and GPx activities, while it contains an abundance of lipids with unsaturated fatty acids that are targets of lipid peroxidation.[3] In addition, the brain glutathione(GSH) concentration is lower than those of the liver, kidney, spleen or small intestine.[5] GSH is a major antioxidant in the brain, with a concentration of approximately 2-3 mM; a much higher concentration than that in blood or cerebrospinal fluid.[6] GSH exerts its action via several mechanisms:

First, GSH non-enzymatically reacts with superoxide[7], nitric oxide (NO) [8] hydroxyl radicals[9], and peroxynitrite ($ONOO^-$).[10] In particular, GSH has a higher ability to scavenge superoxide than N-acetylcysteine or cysteine. [11] Furthermore, there is no known enzymatic defense against hydroxyl radicals, making GSH the only compound capable of scavenging these radicals.[9]

Second, GSH serves as an essential cofactor for a number of enzymes. GSH works as an electron donor for the reduction of H^2O^2 or other peroxides catalyzed by GPx.[12] H^2O^2 is reduced to H^2O by the reaction of GPx with GSH, which is oxidized to GSH disulfide (GSSG).[3] GSSG is then reduced back to GSH, a step catalyzed by GSH reductase with NADPH, and is then reused as a GPx substrate. The relative ratio of the reduced/oxidized forms is over 100 under

normal conditions, but is substantially decreased under stressed conditions.[13] This ratio serves as an indicator of the cellular redox environment.[14] GSH reacts with various endogenous and xenobiotic compounds mediated by glutathione-S-transferase (GST) to form mixed disulfides, which are exported to the outside of the cell.

A third important role of GSH is to serve as a carrier/storage form for cysteine. Cysteine itself has neurotoxic effects mediated by free radical generation, increasing extracellular glutamate and triggering over-activation of N-methyl-D-aspartate (NMDA) receptors.[15] GSH is a non-toxic cysteine storage form with 10-100 times higher concentrations in mammalian tissues than cysteine.[16]

Fourth, GSH is the major redox buffer and maintains intracellular redox homeostasis. Under conditions of oxidative stress, GSH can lead to the reversible formation of mixed disulphides between protein thiol groups, a process critical for preventing irreversible oxidation of proteins.[17]

Fifth, GSH can serve as a neuromodulator/neurotransmitter. GSH binds via its gamma-glutamyl moiety to NMDA receptors.[18] GSH is thought to exert dual (agonist/antagonist) actions on neuronal responses mediated by NMDA receptors in the brain. GSH also serves as an endogenous NO reservoir to form S-nitrosoglutathione (GSNO) [19] GSNO can release NO under certain conditions

with biological effects, while GSNO has a protective effect in brain under oxidative stress conditions.[20]

GSH is a tripeptide consisting of glutamate, cysteine and glycine. The majority of GSH in a cell remains in the cytoplasm, where it is synthesized. GSH synthesis involves two enzymatic steps which involve ATP. Gamma-glutamylcysteine ligase (GCL), also known as γ-glutamylcysteine synthetase, catalyzes the first, (and rate-limiting) enzyme-mediated reaction between glutamate and cysteine, to form γ-glutamylcysteine, which in turn reacts with glycine catalyzed by GSH synthetase(GS) to produce GSH.[3] GSH regulates its own synthesis via feedback inhibition of GCL.[21] Cysteine is the rate-limiting substrate for neuronal GSH synthesis. Neurons rely mainly on extracellular cysteine from astrocytes for GSH synthesis because they lack the means of direct GSH uptake.[22] In the central nervous system, astrocytes store high levels of GSH, with concentrations reaching as high as 8mM.[23] Astrocytes can export their intracellular GSH, mediated by gap junction hemichannels, and continuously re-synthesize GSH.[24] In primary neuron culture, approximately 90% of the total cysteine uptake is mediated by sodium-dependent systems, mainly the excitatory amino acid transporter (EAAT).[25] Neurons utilize cysteine but not cystine for GSH synthesis, whereas glial cells can utilize both.[26] Mitochondria also contain 5-15% of total cellular GSH although they cannot synthesize GSH themselves, as they lack glutamate-cysteine ligase (GCL) activity.[27]

1.2 X-linked Adrenoleukodystrophy

1.2.1 Pathophysiology

X-linked adrenoleukodystrophy (x-ALD) is a peroxisomal disorder caused by mutations in the ABCD1 gene, affecting the function of the encoded protein ALDP, an ATP-binding cassette (ABC) transporter located in the peroxisomal membrane. There are some 1400+ known mutations identified thus far, with 641 of them being nonrecurrent mutations.[28] ALDP deficiency impairs the peroxisomal beta-oxidation of very long-chain fatty acids (VLCFA) and raises cytosolic levels of VLCFacyl-CoA. The VLCFacyl-CoA esters are then further elongated by ELOVL, the human C: 26 specific elongase. This increased substrate availability caused by the primary deficiency in ALDP appears to underlie the enhanced VLCFA elongation and the elevated C:26 plasma levels in X-ALD.[29]

The first cases of X-ALD were probably described in the late 19th century. In 1897 Heubner described a young boy with rapidly progressive neurologic deterioration consistent with X-ALD, classified as having “diffuse sclerosis”.[30] Other cases of “diffuse sclerosis” that resemble X-ALD were also described in 1899 by Ceni and in 1910 by Haberfield and Spieler.[31] Shortly thereafter Schilder suggested that “diffuse sclerosis” was too vague and proposed a more accurate pathological classification of the leukodystrophies. The syndrome of

rapidly progressive cerebral demyelination with inflammatory changes in white matter on autopsy became known as “Schilder’s disease”.[32]

By the early 1970’s, twelve fully documented clinical reports described boys with adrenocortical atrophy and diffuse cerebral sclerosis, along with strong evidence of an X-linked recessive mode of inheritance. Forsyth and colleagues demonstrated an increase in esterified cholesterol in the white matter and cortex of the occipital and parietal lobes of their patients upon autopsy. Based on this finding the authors proposed XALD as an inborn error of metabolism common to the adrenal cortex and the brain.[33] in 1973, with the use of electron microscopy Powers and Schaumburg demonstrated unusual striations in the inner adrenocortical cells which were shown to consist of intracytoplasmic lamellae and lamellar lipid inclusions. Lipid inclusions were also found in testicular cells, Schwann cells and brain microphages from patients with X-ALD.[34] Johnson et al demonstrated that the striated material from adrenal and cerebral white matter was resistant to acetone and ethanol extraction, but readily soluble in nonpolar solvents such as hexane and chloroform, suggesting the accumulation of unusually nonpolar material in these tissues.[35] Subsequently, biochemical analysis of the inclusion bodies revealed that they contained cholesterol, phospholipids and gangliosides esterified with saturated VLCFA.[36] These findings defined X-ALD as a lipid-storage disease and led to the hypothesis that abnormal metabolism of the VLCFA is a key factor in the pathogenesis of X-ALD. Singh and colleagues confirmed this hypothesis by demonstrating that oxidation

of C24:0 and C25:0 is reduced in the fibroblasts from X-ALD patients, whereas oxidation of radiolabeled C16:0 was fully normal in X-ALD cells.[37] In 1984 it was demonstrated by Singh that in rat liver and cultured human fibroblasts, radiolabeled C24:0 is oxidized mainly and possibly exclusively in peroxisomes and that it is this system that is defective in X-ALD. More recently, stable isotope-labeled fatty acids have been used to study fatty acid metabolism and data have confirmed that beta-oxidation of VLCFAs takes place exclusively in the peroxisomes and not in the mitochondria.[38]

The role of VLCFAs in the pathogenesis of X-ALD is largely unknown. VLCFA levels in plasma do not correlate with the patient's phenotype. However, as VLCFA accumulation is the only biochemical abnormality that has been identified in X-ALD, it seems plausible that this accumulation is somehow related to the development of symptoms seen in X-ALD patients. Further support comes from the demonstration of the disruptive effects of C26:0 on cell membrane structure, stability and function [39] and the direct toxic effect of C26:0 on adrenocortical cells resulting in a decreased response to adrenocorticotropic hormone stimulation.[40] In addition,C26:0 accumulation results in oxidative stress[41] and patients with X-ALD show evidence of oxidative stress and oxidative damage, particularly in the adrenal cortex and brain, from lipid peroxidation.[42] A "three hit" hypothesis for the pathophysiology of ALD is proposed by Singh et al. The first hit is the metabolic derangements characterized by excess of VLCFA and oxidative stress. The second hit is the inflammatory disease that results. Finally, the inflammatory

response further causes a generalized loss of peroxisomes/peroxisomal function (third hit), creating a vicious cycle resulting in cell loss and progressive inflammatory demyelinating disease.[43]

1.2.2 Epidemiology

X-ALD has an estimated birth incidence of 1 in 16,800 newborns, both male and female.[44] It is the most common of the peroxisomal disorders and occurs in all regions of the world. [45] As newborn screening for X-ALD has now become technically feasible, true incidence will be available and may, in reality, be higher.

1.2.3 Presentation and clinical course

X-ALD is characterized by the lack of genotype-phenotype correlation. In spite of identical ABCD1 gene mutations, patients can exhibit markedly different neurologic and neuropathologic characteristics. There is evidence that other genetic and/or environmental factors may influence the clinical presentation of X-ALD. Segregation analysis suggests that the phenotypic variation is due to at least one autosomal modifier gene.[46] The symptomatology of X-ALD in children and adults is classified in several phenotypes: Addison-only, Cerebral ALD (cALD) and Adrenomyeloneuropathy (AMN).

Adrenocortical insufficiency or even an Addisonian crisis can be the presenting symptom of X-ALD in as high as 35% of all cases, years or even decades before the onset of neurological symptoms.[47] The recognition of Addison's disease due

to X-ALD has important implications for monitoring and early treatment as well as genetic counseling. It is therefore important to consider X-ALD in any boy or adult male presenting with Addison's disease.

Cerebral ALD in children, adolescence and adulthood are the most rapidly progressive and devastating phenotypes of X-ALD. The cerebral form most often presents in childhood (childhood cerebral ALD, CCALD), usually between the ages of 5 and 12 years, but has not been reported before the age of 2.5 years.[48] CCALD is characterized by progressive cerebral demyelination with a pronounced inflammatory response in the white matter leading to neurodegeneration and death, often before the patient reaches adolescence. Early symptoms include deficits in cognitive abilities that involve visuospatial and visuomotor functions or attention and reasoning. The initial symptoms result in decline in school performance. These early clinical symptoms are often misdiagnosed as attention deficit hyperactivity disorder, and can delay the diagnosis of CCALD. As the disease progresses, more overt neurologic deficits become apparent, including withdrawn or hyperactive behavior, apraxia, auditory impairment, decreased visual acuity, hemiparesis, cerebellar ataxia and seizures. At this stage progression is extremely rapid and devastating. Affected boys can lose the ability to understand language and to walk within a few weeks. Eventually, patients are bedridden, blind, unable to speak or respond, requiring full-time nursing care and feeding by nasogastric or gastrostomy. Usually death occurs two to four years after onset of symptoms, or, if well-cared for, patients may remain in this apparent vegetative state for several years.[49] Less frequently,

cerebral demyelination as the presenting phenotype of X-ALD occurs in adolescence (AdolCALD) or adulthood (ACALD). The symptomatology in these patients resembles CCALD, but the initial progression of symptoms is usually much slower. In adults, early cognitive decline is rarely recognized by family or coworkers. As the disease progresses, psychiatric disturbances mimicking schizophrenia or psychosis are common.[50] In these cases, the diagnosis of X-ALD is often delayed, particularly when no family history of X-ALD is present and when clinical symptoms of Addison's disease are absent.

Approximately 10% of boys or adolescents with cerebral ALD may not develop the rapidly progressing neuroinflammatory stage of the disease. The same may occur in men with ACALD or in men with AMN who develop secondary cerebral demyelination. This cerebral demyelinating form of X-ALD is often referred to as "chronic or arrested cerebral X-ALD." The cerebral demyelinating process arrests spontaneously and the patient can remain stable for a decade or even longer. But even after a 10-15 year period of stability, sudden onset of rapid neurologic deterioration may occur, reflecting a full progression to the neuroinflammatory stage of the disease.[31]

Virtually all patients with X-ALD who reach adulthood will develop AMN, usually in the third or fourth decade. Patients develop gradually progressive spastic paraparesis, sensory ataxia with impaired vibration sense, sphincter dysfunction, pain in the legs and impotence.[51] The pathological basis of AMN is a

noninflammatory distal axonopathy that involves the long tracts of the spinal cord, and to a lesser extent, the peripheral nerves. This phenotype is most often slowly progressing, causing severe motor disability of the lower limbs over one to two decades but mild or no significant deficits in the upper limbs or hands.[31] Approximately 20% of AMN patients develop cerebral demyelination, according to a 10-year retrospective study.[52] After an initial progression, the demyelinating lesions can stabilize spontaneously, leading to moderate cognitive deficits. However, once the cerebral demyelinating lesions have entered the active phase of neuroinflammation, the prognosis is as poor as in CCALD.[53]

Unlike many x-linked diseases where female carriers remain asymptomatic, many women with X-ALD develop AMN-like symptoms. The onset of neurologic symptoms occurs mainly between the fourth and fifth decade and they are very similar to those observed in adult males with AMN. Sensory ataxia, fecal incontinence and pain in the legs are more prominent in symptomatic women with AMN. Cerebral involvement and adrenocortical insufficiency are rare, 2% and 1% respectively.[49]

X-ALD phenotypes are not static. Presymptomatic males are nearly all at risk to develop neurologic disease (cALD or AMN), or endocrinologic symptoms (Addison's disease). Addison-only males can develop AMN or cerebral ALD, and AMN males can develop cerebral demyelination. Presymptomatic women with

X-ALD are at risk to develop AMN. Progression of X-ALD in a specific individual cannot be predicted.

1.2.4 Diagnosis and Management of X-ALD

Newborn screening based on the measurement of C26:0 lysophosphatidyl-choline (26:0-lyso-PC) in dried blood spots [54] is now technically feasible, but is not widely used. This screening could lead to identification of pre-symptomatic patients with X-ALD, but its implementation is a matter of public policy and ethical considerations are still being discussed.

If X-ALD is suspected in a male with neurological symptoms or Addison's disease, VLCFA elevation in plasma is the first step in making the diagnosis. Since increased plasma VLCFA are not pathognomonic for X-ALD, if metabolic screening reveals increased VLCFA, the next step is to confirm the diagnosis by performing ABCD1 mutation analysis. For women with X-ALD, the diagnostic test of choice is mutation analysis, as about 15% of women with X-ALD have normal plasma VLCFA levels.[55] Family screening should be done by mutation analysis as well.

Boys or men who do not have Addison's disease should be evaluated yearly by an endocrinologist for adrenocortical dysfunction. Steroid (ACTH) replacement can be initiated if necessary.

Boys aged 3-12 years old without neurological deficits should be monitored every 6 months with magnetic resonance imaging (MRI) for signs of cerebral ALD. About 80% of boys with cALD display the posterior pattern of demyelination on MRI. The lesion starts in the splenium of the corpus callosum and spreads out into the parieto-occipital white matter. In approximately 10-20% of boys with inflammatory cerebral involvement, the process is reversed and starts in the frontal regions with concomitant involvement of the rostrum and genu of the corpus callosum.[55] The 34-point severity score has been developed by Loes et al. for use in monitoring location, extent of involvement and presence of focal and/or global atrophy with each 6 month MRI.[56] High-field magnetic resonance spectroscopy (MRS) studies have shown that abnormal reductions in N-acetyaspartate:choline ratio precedes the development of changes on MRI.[57] These changes could prove useful in determining when to move to treatment with limited available therapies for cALD.

Allogeneic hematopoietic stem cell transplantation (HCT), despite its significant mortality risk, is the only known therapeutic intervention which can arrest the progression of cerebral demyelination in X-ALD.[58] If performed early, when affected males have no or minor symptoms due to the cerebral demyelinating disease, HCT demonstrates excellent likelihood of survival with good neurologic and neuropsychological function in most boys. However, once pronounced cerebral involvement exists along with neurological deficits, HCT is often not successful in prolonging survival or preserving function.[59] Based on evidence that

oxidative stress and oxidative damage contribute to the central nervous system damage seen in X-ALD, Tolar et al demonstrated improved survival using adjuct N-acetylcysteine (NAC) therapy in boys undergoing HCT for advanced cerebral X-ALD.[60] This work will be discussed in a later chapter. Recently, transplantation with autologous bone marrow transfected in vitro with ABCD1 has been performed with success. So far, bone marrow transplant has only been reported in children with cerebral ALD. There is little reason why it should not be equally effective in adults. Preliminary experiences from transplant centers in Germany and France show survival rates comparable to those in childhood cerebral ALD; however, pre-existing severe AMN symptoms such as motor disability or urinary incontinence seriously influence the clinical outcome after transplantation.[61]

For adult men with or without signs of AMN, yearly evaluation by a neurologist is recommended. Since adult men can develop cerebral ALD, an MRI is usually performed. There is no proven treatment for cerebral ALD in adults, but studies of HCT have included very small numbers of adult men. For AMN there is no effective disease modifying therapy available yet. Lorenzo's Oil (LO), a mixture of glycerol trioleate and glycerol trierucate oils, showed great promise, but open-label trails have shown that the disease progresses even when plasma VLCFA are normalized by LO treatment.[62, 63] A large randomized placebo-controlled clinical trial was designed to provide a definitive answer, but was unfortunately aborted before completion by the safety monitoring board because of presumed side effects of the placebo treatment. There is also a retrospective study

suggesting that if presymptomatic boys are started on LO, it may delay the onset of neurological symptoms.[64] Currently, Lorenzo's oil is available through expanded access to males with x-linked adrenoleukodystrophy, as determined by biochemical or genetic determination, between the ages of 18 months to 18 years who have a normal cerebral MRI at baseline.[65]

Lovastatin also lowered plasma VLCFA [66], but a placebo-controlled trial revealed that lovastatin did not have an effect on the C26:0 level in peripheral blood lymphocytes and erythrocytes nor on the VLCFA content of low-density lipoprotein fraction.[65] Antioxidants reduce markers of oxidative stress and axonal degeneration in the spinal cord of Abcd1 knockout mice.[67] Based on this observation, a clinical trial with anti-oxidants in AMN is ongoing in Spain.[68] Bezafibrate has been shown to decrease VLCFA in X-ALD fibroblasts by direct inhibition of fatty acid elongation activity.[69] A proof of principal trial showed that bezafibrate was unable, at least in the doses given, to lower VLCFA levels in plasma or lymphocytes in patients with X-ALD.[70]

1.3 Parkinson's disease

1.3.1 Pathophysiology

Parkinson's disease (PD) is typically a late-onset neurodegenerative disorder characterized by difficulty initiating movement, rigidity, tremor and postural instability. While motor dysfunctions represent the most often seen clinical features of the disease, non-motor symptoms such as sleep disturbances,

dementia and depression often also occur. Motor disturbances are produced predominantly by the degeneration of dopaminergic neurons of the substantia nigra and their projections to the striatum, although other neuronal populations are affected in the disease.

In recent years, strides have been made in understanding the initiating causes of familial PD. Genetic mutations in several genes including α-synuclein, Parkin, UCH-LI, DJ-1, PINK-1 and LRRK2 have been identified, providing hope for potential therapeutic interventions.[72,73] Alpha-synuclein is the major protein component of Lewy bodies, the pathological hallmark not only in the brains of mutation carriers, but also in the common sporadic for of PD. Genome-wide association studies have revealed that genetic variants in the α-synuclein gene represent the most consistent risk factor for PD across different populations. Familial PD, however, accounts for only 5-10% of the all Parkinson disease. Although genetics have not revealed an underlying cause for sporadic or idiopathic PD, growing evidence implicates other biochemical abnormalities including: oxidative stress, mitochondrial dysfunction, proteasomal dysfunction and GSH depletion. Oxidative stress can induce mitochondrial DNA mutations, damage the mitochondrial respiratory chain, alter membrane permeability and influence calcium ion homeostasis and mitochondrial defense systems. Once damaged, mitochondrial DNA can amplify oxidative stress by decreased expression of critical proteins important for electron transport, leading to a vicious cycle of ROS and organelle dysregulation that eventually triggers apoptosis.[74]

Dysfunction of molecular and organelle degradation pathways is a further hallmark of PD and increasing evidence indicates functional interactions between the ubiquitin proteasome system and autophagy.[75,76] While both degradation systems are involved in the clearance of misfolded proteins, a special form of autophagy also known as mitophagy removes defective mitochondria from cells.[77] This clearance process is controlled via PINK1 and Parkin.[78] In PD, the dysfunction of these clearance systems facilitates the accumulation of α-synuclein and defective mitochondria. A positive feedback loop, which turns seemingly mild dysfunctions in misfolded protein handling into a self-perpetuating cycle, is indicated by the finding that mutated forms of α-synuclein may inhibit their own degradation via chaperone-mediated autophagy.[79] Although the cause or consequence of each of these in relation to PD is unclear, most evidence points to GSH depletion as an early forerunner to the cascade of events leading to the loss of dopaminergic neurons.

1.3.2 Epidemiology

Parkinson's disease is the second most common neurodegenerative disease worldwide, affecting 0.3% of the general population, and 1% of the population older than 60 years. At the age of 80, the prevalence rises to 3%.[80] Mean age of onset is 65 years, with those diagnosed early in adulthood typically having slower disease progression than those diagnosed later in life.[81] The ratio of men to women affected is 1.5:1.[82] Idiopathic Parkinson disease is much more common than hereditary PD, occurring in about a 90:10% ratio.[83] PD protective factors

include cigarette smoking and high coffee consumption. Risk factors include a family history of PD, pesticide exposure and head injury. Constipation was thought to be a risk factor for PD, but is now thought to be an early symptom of the disease.[84] To date, aging represents the most significant risk factor for developing PD.[85]

1.3.3 Presentation and Clinical Course

The clinical syndrome of idiopathic PD is characterized by bradykinesia, resting tremor, rigidity and postural instability. In addition to these motor symptoms, many patients experience a wide range of non-motor symptoms that sometimes even precede the typical movement disorder, such as hyposmia, sleep disturbances such as REM sleep behavior disorder, fatigue, depression, constipation and other dysautononmic symptoms.[86]

The course of PD varies widely, but appears to occur in three major clinical subgroups: tremor dominant (8%), akinetic-rigid (26%) and mixed, which is the most prevalent (66%).[87] Late symptoms, occurring by approximately 15 years after symptom onset, include freezing, postural instability, falls and dysphagia. Psychiatric and autonomic disturbances typically occur 5-10 years after symptom onset and include anxiety, postural lightheadedness, sialorrhea, urinary urgency, nocturia, and sexual dysfunction. The likelihood of cognitive impairment increases with time after symptom onset. Approximately 35 % of patients experience mild cognitive impairment at diagnosis and 50% after 5 years from

diagnosis. Approximately 80% or greater will experience dementia 20 years after diagnosis. [88-92]

1.3.4 Parkinson disease management

There are no established disease-modifying or neuroprotective therapies for PD. Medications for symptom management are usually initiated when patients experience functional impairment or social embarrassment from their symptoms. If motor symptoms are mild but require therapy, a monoamine oxidase type B inhibitor (MAOBI; selegiline or rasagiline) may be tried before moving to more potent treatment such as a dopamine agonist or levodopa. A disease-modifying effect of rasagiline has been reported, but has not been confirmed by other clinical trials.[93] Anticholinergic medications have been shown to be effective for improving motor function in PD, but data on their benefits for tremor were inconclusive.[94] Older studies suggest that β-blockers such as propanolol may improve parkinsonian tremor and motor function.[95] Clozapine has been shown to improve Parkinson disease tremor, but is generally reserved for bothersome or disabling tremor resistant to other therapies.[96] Evidence supporting amantadine for treatment of Parkinson's disease is mixed, however expert opinion from the International Parkinson and Movement Disorder Society and European Federation of Neurological Societies concluded that amantadine is likely efficacious for symptomatic monotherapy and adjunct therapy. For those with more severely impaired activities of daily living, levodopa or a dopamine agonist is usually initiated. Multiple large clinical trials demonstrate that levodopa

provides the greatest symptomatic benefit for Parkinson disease and is associated with less freezing, somnolence, edema, hallucinations and risk of impulse control disorders than dopamine agonists.[97] Dopamine agonists are also effective in early Parkinson disease and are less likely than levodopa to cause dopaminergic motor complications, particularly dyskinesia. Because younger age-at-onset of Parkinson disease is a risk factor for dyskinesia, dopamine agonists are usually introduced as initial treatment for patient younger than 60 years.[98]

Managing motor fluctuations, including symptom reemergence between medication doses can be difficult in some patients with Parkinson disease. Strategies for reducing the time that medication is not optimally effective ("off" time) include increasing the dosage of dopaminergic medication, adding another dopaminergic medication, dividing the levodopa dosage into smaller but more frequent doses (levodopa dose fractionation), or adding a catechol-O-methyltransferase inhibitor (COMTI) or MAOBI to inhibit the breakdown of levodopa and dopamine and prolong their effects.[99] There is extensive but conflicting evidence for the use of selegiline to reduce off time.[100,101] Dopamine agonists may be added to levodopa to reduce off time. Adjunctive pramipexole, ropinirole and transdermal rotigotine have all shown a significant reduction in off time compared with placebo in well controlled studies. Intermittent, as needed, subcutaneous apomorphine provides rapid delivery to reduce bothersome off periods, but may increase dyskinesia.[102] A study of levodopa-carbidopa intestinal

gel, administered directly into the duodenum by pump through a gastrostomy catheter, confirmed earlier small, open-label studies showing a marked reduction in off time.[103]

Treatment of severe or bothersome dyskinesia may involve dopaminergic medication reduction, but this will typically often worsen parkinsonism. Amantadine is frequently used for reducing dyskinesia severity and duration, and is typically well tolerated.[104] Levodopa-carbidopa intestinal gel may be useful in the future and exploratory trials of other agents are ongoing.

Other medication adverse effects such as nausea, psychosis, impulse control disorders, dopamine dysregulation syndrome and punding all pose additional challenges in the treatment of PD. Nausea is a frequent, generally transient adverse effect of dopaminergic therapy. Slow titration of dopaminergic therapy and medication administration with food can reduce nausea; however, food may delay gastric emptying and dietary protein may interfere with levodopa absorption. An additional dose of carbidopa (which inhibits peripheral conversion of levodopa to dopamine) 30 minutes before dosing levodopa, may prevent levodopa-induced nausea, but not dopamine-agonist-induced nausea. [105] Medications such as domperidone, a peripheral dopamine D2-receptor agonist may reduce nausea from dopaminergic medications, but is still unavailable in the U.S.[106] Impulse control disorders (ICDs)are typically, but not entirely, associated with dopamine agonist use.[107] A history of obsessive-compulsive disorder,

impulsive personality, or addictive behaviors increases the likelihood of ICDs. Dopamine agonist dose reduction or discontinuation, offset by increasing levodopa, in generally effective treatment in ICDs. Withdrawal symptoms may include anxiety, depression, fatigue, pain, orthostatic hypotension, and drug cravings (dopamine agonist withdrawal syndrome), and may be unresponsive to increasing levodopa in 15-20% of patients.[108] Management of dopamine dysregulation syndrome (DDS) typically involves a gradual reduction of levodopa and immediate discontinuation of "booster" doses of medications such as subcutaneous apomorphine boluses or rapid-acting levodopa formulations.[109] No studies have formally examined the management of DDS, but a small case series of 4 PD patients reported a response to valproate in all 4 patients.[110]
Punding, defined as repetitive, often purposeless, stereotyped behaviors such as sorting or disassembling, occur in up to 15 % of Parkinson patients. Management of this adverse effect may include reduction or cessation of dopaminergic medications. A small open-label study indicated that amantadine and quetiapine may also be beneficial. [111]

Hallucinations are both a feature of later-stage Parkinson disease and a consequence of Parkinson disease medications. Additional psychotic symptoms however, are generally thought to be drug-related. Clozapine and quetiapine have been most extensively studied for the treatment of psychosis in Parkinson disease, since other neuroleptics have the propensity to worsen parkinsonism. Clozapine is consistently efficacious in a number of studies, whereas the results

for quetiapine as mixed.[112,113] Quetiapine is prescribed first most often because of the risk of agranulocytosis and the requirement for frequent blood monitoring with clozapine. Treatment approaches to new-onset or increased hallucinations include initial exclusion of systemic illness, such as infection or other medication use. Subsequent reduction or discontinuation of antiparkinsonian drugs is done in order from lowest efficacy, starting with anticholinergics, amantadine, and MAOBIs, followed by dopamine agonists and COMTIs[114] Finally, the levodopa dose is cautiously reduced and the patient is monitored for a disabling increase in parkinsonism, including the rare occurrence of neuroleptic malignant like state. Again, clozapine clearly has the highest efficacy for hallucinations and psychosis. A recent study showing efficacy for the selective 5-HT2A inverse agonist pimavanserin for psychotic symptoms provides new hope for therapies that do not require extensive monitoring.[115]

The management of non-motor symptoms is another challenge in the treatment of Parkinson disease. Rapid eye movement sleep behavior disorder, depression and cognitive impairment are a few of these troublesome effects of PD. Clonazepam is a first-line therapy for rapid eye movement sleep behavior disorders (RBD) in general, but only a few case reports and case series are reported on its use in PD patients. There is similarly little evidence for the use of melatonin in RBD specifically in PD, but it may be an alternative to clonazepam for those patients in whom clonazepam is contraindicated such as those with

dementia, obstructive sleep apnea, or extreme frailty with an increased risk of falls.[116]

The literature on the management of depression in PD is mixed. A 2013 systematic review found no statistically significant superiority of antidepressants compared with placebo, as a group or by class, but tricyclic antidepressants were superior to selective serotonin reuptake inhibitors.[117] Tricyclic antidepressants were found to be superior to placebo in other studies, but their use may be limited due to concerns about the adverse effects in older patients who are cognitively impaired.[118]

PD management represents a considerable challenge to the medical community, and more importantly to those who suffer from its effects. Given the progressive nature of this disease, and its multifaceted disability, the greatest unmet therapeutic need is the identification of effective neuroprotective and disease modifying therapies.

1.4 Gaucher disease

1.4.1 Pathophysiology

Gaucher disease (GD), the most common autosomal recessive lysosomal storage disease, is caused by a deficiency or absence of the activity of the enzyme glucocerebrosidase, which breaks down the glycolipid glucocerebroside into glucose and ceramide inside the lysosome. Glucocerebrosidase is encoded

by the glucocerebrosidase (GBA1) gene. Mutations in the GBA1 cause glucocerebrosidase deficiency and the subsequent accumulation of the undegraded substrate glucocerebroside inside the lysosome of cells composing the reticulo-endothelial system. This accumulation results in Gaucher disease.[118] The main biological function of macrophages is phagocytosis-mediated breakdown of aging cells such as erythrocytes, which have glucocerebroside-rich membranes. Gaucher disease macrophages that have accumulated glucocerebroside appear engorged and are often referred to as “Gaucher cells”. Gaucher cells primarily populate the spleen, liver and bone marrow, resulting in inflammation and organomegaly.[119]

1.4.2. Epidemiology

Gaucher disease affects men and women equally. According to a report by the National Organization for Rare Disorders, the GD incidence rate may be as high as 1 in 450 births among individuals with Ashkenazi Jewish ancestry and 1:20,000 to 1:200,000 in the general population.[120] The National Gaucher Foundation estimates the incidence of GD type 1 (GD1) in the U. S. to be about 1 in 20,000 live births or a prevalence of about 1 in 40,000. [121] A high prevalence of GD1, especially with mutations N370S and 84GG, is seen among Ashkenazi Jews, whereas mutations in N370S are found among North American, European and Israeli populations. GD1 occurs mainly in adults and is the most frequent type, accounting for 95% of GD cases. GD type 2 (GD2) is rare, occurring in fewer than 1 in 100,000 people and generally affects infants 4-5 months old.[122]

GD type 3 (GD3) is also a rare form that affects fewer than 1 in 100,000 people.[123]

1.4.2 Presentations and Clinical Course

GD is classified into GD1 (non-neuropathic), GD2 (acute neuropathic), and GD3 (chronic neuropathic), according to the presence of neurological deterioration, age at identification and disease progression rate. If GD onset occurs prior to adulthood, a more rapidly progressing disease is suspected. GD1 is associated with visceral complications without CNS involvement. However, the wide spectrum of clinical manifestations seen in patients with type 1 GD has led many to argue that this classification system is too simplistic. Indeed the observation of parkinsonism, dementia and subclinical peripheral neuropathy in patients with type 1 GD has challenged the more traditional definition as non-neuronopathic. Initial manifestations normally begin with splenomegaly, hepatomegaly, anemia, leukopenia and thrombocytopenia.[124] Further progression involves gastrointestinal complications such as portal hypertension, cirrhosis, ascites, esophageal hemorrhage, and bone lesions manifested in chronic bone pain, skeletal deformities, osteonecrosis, osteopenia, and osteoarticular infections.[125]. GD2 and GD3 are neuronopathic variants with several distinguishing characteristics. GD2 involves the brain, spleen, liver, and lungs, with severe neurological complications. The disease progresses rapidly, leading to death within the first 2 years of life.[126] GD3 has only mild visceral manifestations but causes severe, progressive myoclonic seizures, which can lead to death within

the first 2 decades. GD3b involves more visceral features, such as massive hepatosplenomegaly, growth retardation and supranuclear gaze palsy.[127] GD3c patients with a specific genetic mutation (D408H) will often die in early adulthood due to a rare cardiac mitral and aortic calcification.[128]

GD1 is classified according to clinical severity scores using a scoring system: Gaucher Disease Severity Score Index, Type 1 (GauSSI-1). This severity score allows for a more thorough and reliable method to correlate the differences in genotypes and phenotypes of the patients, to correlate patient response to biological markers and to account for the variability in clinical response and severity of disease. There are six domains to score in the GauSSI-1: skeletal, hematological, biomarker, visceral, lung, and neurological. The severity of symptoms or other objective measures are scored with a possible 42 points with higher scores reflecting more severe GD. [129]

Among the neurological complications of GD1 is parkinsonism. This has challenged the traditional classification of type 1 GD as non-neuronopathic, as mentioned earlier. The possibility of a link between the two diseases was first suggested in the clinic setting where a small number of case reports described parkinsonism features in GD patients.[130] Larger studies have since been published which have confirmed these findings.[131] Heterozygote carriers of mutations in the BGA gene also have an increased frequency of Parkinson disease, and approximately 5-10% of PD patients have GBA mutations,

confirming mutations of this gene as numerically the most important genetic predisposing risk factor for PD identified to date.[132] Data on the penetrance and lifetime relative risk (RR) of PD in patients with GD are conflicting. Some studies report a slightly higher estimated penetrance and lifetime RR of PD in heterozygous GBA mutation carriers compared to GD patients, but this may reflect differences in size of the cohorts evaluated, rather than a significant difference in the overall risk of PD between the two groups. Studies have investigated the frequency of GBA mutations in patients with sporadic PD and have shown that the odds ratio for a known patient with idiopathic PD to harbor one GBA mutation is 5.4.[133] Additionally, when compared to other PD-related genes such as α-synuclein and parkin, the frequency of GBA mutations is higher in individuals with parkinsonism.[134] Of further interest is a recent study that identified a GBA mutation (E326K) that can predispose to PD but is a variant that does not, when homozygous, cause PD.[135]

To date, over 300 GBA1 mutations and polymorphisms have been reported, but correlations between the clinical phenotype and molecular genotype remain limited. Studies have shown that patients with identical genotypes can have vastly different clinical manifestations and severity, even between siblings and twins, whereas patients with different molecular genotypes can share similar clinical phenotypes. [136] Furthermore, there is not a strong correlation between the amount of accumulated substrate and/or residual glucocerebrosidase enzyme activity and clinical manifestations in patients. This suggests that Gaucher

disease is not a “simple” monogenic disorder, and that additional factors such as epigenetics and/or genetic modifiers may contribute to disease development and phenotype.[137]

1.4.3 Diagnosis and Management of Gaucher disease

GD is often diagnosed during initial clinical examination by the presence of unexpected anemia, thrombocytopenia and organomegaly. Clinical diagnosis is confirmed by biochemical diagnosis. Detection of low enzymatic activity of GBA in the peripheral blood compared with normal control is still the gold standard for diagnosing GD.[138]

Therapeutic treatment options in GD consist of enzyme replacement therapies (ERT) and substrate reduction therapy (SRT). The first ERT was introduced in 1991. The placental-derived macrophage-targeted glucocerebrosidase, alglucerase (Ceredase, Genzyme Corp.) led to the revolution in GD management and this finding also led to the use of ERT for other lysosomal storage disorders. In 1994 imiglucerase (Cerezyme, Genzyme Corp.) was approved by the FDA. Imiglucerase is an analog of glucocerebrosidase produced by DNA technology using Chinese hamster ovary cells, and subsequently replaced alglucerase. In June of 2009, Genzyme Corporation announced a viral contamination at its manufacturing site, and the dramatic reduction in available supply to 20% left the ERT unavailable to many patients worldwide. This shortage of ERT stimulated interest in the development of 2 new ERTs.

In 2010, velaglucerase alfa (VPRIV, Shire Human Genetics therapies, Inc), an analogue of recombinant glucocerebrosidase produced in human fibroblast cell lines, became the third ERT approved by the FDA.[139] In May 2012, FDA approved taliglucerase alfa (ELEYSO, Pfizer Inc.), which is produced genetically by modified carrot cells. The availability of a plant-cell-derived system to manufacture taliglucerase alfa offers the possibility of large-scale production of the enzyme at a lower cost and lowered potential for shortages. All three ERTs currently available are administered every other week via intravenous injection. Common hypersensitivity reactions and production of antibodies may occur with all available ERTs. All are incapable of crossing the blood-brain barrier and are specifically indicated for moderate to severe GD1 treatment.[140]

In contrast to ERT, which aims to replace the defective enzyme with active enzyme, SRT targets the biosynthetic pathway and reduces the load of glucocerebroside influx into the lysosome. Miglustat (Zavesca, Actelion Pharmaceuticals Limited) is a synthetic D-glucose analogue which works by inhibiting the enzyme glucocerebrosidase, the enzyme responsible for glucocerebroside synthesis and other glycosphingolipids, thus reducing it to residual activity and preventing its influx into the lysosome. The drug was FDA approved in 2003 for the treatment of mild to moderate GD3, and is orally administered three times daily. Since Miglustat is capable of penetrating the blood-brain barrier, it was intended as a prototype for the management of the neuronopathic forms of GD. Although this SRT offers more convenient dosing

than available ERTs, clinical trials with miglustat in GD3 patients showed no improvement in neurological symptoms. The drug also caused a higher incidence of adverse reactions such as tremor, weight loss, reduced platelet counts, numbness and feeling of burning on the hands and feet. Moreover, long-term reductions of glycosphinglolipids could affect a variety of cellular functions because of the essential roles of these lipids.[141] Miglustat is now approved in the U.S. for patients who cannot take ERT because of anaphylactic reactions. Another SRT, eliglustat (Cerdelga, Genzyme Corp.) was just recently FDA approved for select GD1 patients. This drug is predominantly metabolized by CYP2D6, and metabolizer status must be taken into consideration when dosing this therapy. The FDA indication is for the long-term treatment of adult patients with Gaucher disease type 1 (GD1) who are CYP2D6 extensive metabolizers (EMs), intermediate metabolizers (IMs), or poor metabolizers (PMs) as detected by an FDA-cleared test.[142] Because eliglustat does not penetrate the blood-brain barrier, it may not add value in the treatment of neurologic symptoms of GD.

In addition to ERT and SRT, other supportive management strategies are necessary to alleviate the disease symptoms such as bone disease, hepatosplenomegaly, bleeding, pulmonary hypertension, seizures, and parkinsonism. Treatment of bone disease with bisphosphonates is usually necessary. In severe thrombocytopenia or symptomatic organomegaly unresponsive to ERT, splenectomy might be necessary. Defects in platelets, coagulation and non-corrected thrombocytopenia pathways may cause increased

bleeding risk in GD patients and require constant monitoring.[143] In patients with moderate to severe GD with life-threatening complications such as hepatopulmonary syndrome and pulmonary hypertension, higher doses and longer treatment with ERT may be needed along with adjuvant therapy such as vasodilators and/or warfarin. For visceral and neurologic complications, the best option currently is higher doses of ERT. For those GD patients who develop Parkinson disease, improvements and worsening of symptoms may occur despite optimized ERT therapy.[144] Because of the severity and complexity of GD, healthcare providers need to individualize treatment options, as limited standardized treatment guidelines are available.

In recent years a few studies have shown promising results using gene therapy and chaperone treatment in animals. Studies using murine GD1 models injected with lentiviral and null mice GD models injected with adeno-associated viral vector harboring the human GBA1 gene have shown promising results. [145,146] These vectors induce the liver to secrete GBA1 in young animals and older mice with GD. Abnormal protein folding is thought to be a mechanism in many inherited diseases, and chaperone therapy is based on the ability of small molecules to interact with mutant proteins that are misfolded. Chaperone molecules bind to GBA1 during biosynthesis, then dissociate from the enzyme for delivery to the lysosome, allowing BGA1 to be delivered to the normal site. So far, studies of chaperone therapies in GD have shown disappointing results, but may be an option for combination treatment strategies in the future. [147]

CHAPTER 2

ANTIOXIDANTS IN NEURODEGENERATIVE DISORDERS

2.1 Select antioxidants previously studied in neurodegenerative disorders

2.1.1 Vitamin E

The nervous system is vulnerable to the damaging effects of highly reactive free radicals for several reasons. The brain contains high amounts of polyunsaturated fatty acids that are susceptible to lipid peroxidation, receives a large percentage of oxygen, and is relatively deficient in certain antioxidant enzymes. In addition, specific regions of the brain have high iron concentrations. Thus, antioxidant defenses are critically important to protect the brain and neural tissues from oxidative damage. Neuroprotection by antioxidants such as vitamin E has therefore drawn much interest. Vitamin E is the major lipid-soluble, chain-breaking antioxidant in the body, protecting the integrity of membranes by inhibiting lipid peroxidation. Mostly on the basis of symptoms of primary vitamin E deficiency, including peripheral neuropathy, it has been demonstrated that vitamin E has a central role in maintaining neurologic structure and function.[148] Since orally supplemented vitamin E reaches the cerebrospinal fluid and brain, it has been extensively studied for its antioxidant effects.[149] Several animal studies have shown vitamin E to be neuroprotective against free radical-mediated injury, protect neurons of the locus coeruleus from death in an early model of PD,[150] prevent the toxin-induced destruction of striatal dopaminergic terminals,[151] and regulate the levels of antioxidant defenses such as glutathione and superoxide dismutase.[152] These preclinical studies prompted a large, multicenter study of vitamin E in slowing the progression of PD.

In 1987, the DATATOP (Deprenyl And Tocopherol Antioxidative Therapy Of Parkinsonism) clinical trial was initiated under the primary sponsorship of the National Institute of Neurological Disorders and Stroke (NINDS) of the National Institutes of Health. Deprenyl (selegiline), a type-B monoamine oxidase inhibitor, and a-tocopherol, the biologically active antioxidant of vitamin E, were hypothesized to slow the clinical decline in patients with early and otherwise untreated PD. The DATATOP trial was based on the assumption that deprenyl or tocopherol, alone or in combination, would slow disability over a 2-year period of observation. DATATOP was designed as a 2 X 2 factorial, double-blinded, placebo-controlled multicenter trial to determine if deprenyl 10 mg/day and/or tocopherol 2,000 IU/day, administered to otherwise untreated patients with early PD, would prolong the time until levodopa therapy was judged necessary to treat emerging disability. Between September 1987 and November 1988, 35 Parkinson Study Group (PSG) investigators enrolled 800 patients with early, untreated PD from 23 research sites in the United States and five in Canada. After baseline evaluation and randomization to one of four treatment assignments (double-placebo, active deprenyl alone, active tocopherol alone, both active deprenyl and tocopherol), subjects were re-evaluated at 1 and 3 months and then at approximately 3-month intervals with respect to standardized clinical measures, including the Unified Parkinson Disease Rating Scale (UPDRS). The primary endpoint of the study was functional disability sufficient to warrant the initiation of levodopa therapy. [153] On the basis of follow-up of the initial 14 ± 6 (mean ± SD) months of the DATATOP trial, the beneficial effects of

deprenyl were confirmed and occurred largely during the first 12 months of treatment and significantly delayed the onset of disability that would require levodopa therapy (hazard ratio 0.50; 95% CI, 0.41-0.62; $p < 0.001$). The difference in the estimated mean time to the end point of disability was about 9 months, when subjects assigned to deprenyl were compared with those not assigned to deprenyl. The study found no beneficial effect of tocopherol and no interaction between tocopherol and deprenyl.

Despite these results, vitamin E has been studied in conjunction with other antioxidants in PD, including vitamin C and more recently, coenzyme Q10, which will be discussed in subsequent sections of this chapter.

2.1.2 Vitamin C

Vitamin C (ascorbic acid) is a required nutrient for a variety of biological functions. Humans and other primates have lost the ability to synthesize ascorbic acid due to a defect in L-gulono-1, 4-lactone oxidase, an enzyme that catalyzes the conversion of L-gulonolactone into ascorbic acid.[154] Humans, primates and a few other animals depend on the diet as a source of vitamin C to prevent disease and maintain general health. For many years it was widely believed that the only function of ascorbate or vitamin C in the body was to promote collagen synthesis and that its only role in therapeutics was to treat scurvy. In the late 1990's, it became evident that ascorbate had important functions in the body, particularly in the brain.[155] Ascorbate concentrations in the CSF of about 200µM have been reported, in contrast to plasma concentrations of

50 µM or less. It appears that much of the ascorbate in brain is in the neurons themselves. High ascorbate concentrations have been localized in the neuron-rich areas of the hippocampus and neocortex in human brain, where the ascorbate content in as much as two-fold higher than in other brain regions.[156] The major route by which ascorbate enters the CNS involves transport from plasma to the CSF across the epithelium of the choroid plexus.[157] Two ascorbate transporters, first cloned in 1999, are known thus far: sodium-dependent vitamin C transporter type 1 (SVCT1), which resides primarily in the brush-border membranes of intestinal and renal tubular cells, and SVCT2, which is found in most other tissues, including the brain.[158] Once in the CSF, ascorbate enters the brain interstitium via diffusion.

The functions of ascorbate in the CNS and brain are numerous, but incompletely understood. Ascorbate acts directly to scavenge oxygen or nitrogen-based radical species generated during normal cellular metabolism. It can prevent lipid peroxidation, especially in combination with vitamin E, and acts to recycle vitamin E in membranes. Ascorbate is thought to have an effect on neural maturation and has been proposed to have function as a neuromodulator of both dopamine and glutamate-mediated neurotransmission.[159] There is also evidence that ascorbate is involved in the regulation of both acetylcholine and catecholamine release form synaptic vesicles.[160]

In 1983 Hoffer proposed that ascorbate might be useful as a protection against auto oxidation damage in critical brain areas.[161] This work prompted interest in the use of vitamin C in Parkinson's and Alzheimer's diseases. In 1979 Fahn and others form the movement disorders specialist's community started to consider the endogenous toxin hypothesis as a plausible mechanism for the causation of PD. They began to study whether high dosages of lipid and water soluble antioxidants might slow the progression of the disease by increasing the scavenging of oxyradicals in the brain. In a small open-label trial, patients with a diagnosis of early PD who had not yet started on levodopa therapy, were started on vitamin E (α-tocopherol), 3200mg and vitamin C, 3000mg daily in four divided doses. Progression of their PD was measured using the Columbia PD Rating Scale and the Schwab-England Activities of Daily Living Scale. Patients were started on levodopa therapy only when symptoms become a threat to occupational, physical, or social disability. The primary endpoint of the study was time to required levadopa or a dopamine agonist. The mean ±SEM duration of PD before starting dopaminergics was 71.9 ±6.5 in the group with age of onset of PD <54 years. The mean ± SEM duration of PD before starting dopaminergics was 63.3 ± 3.9 in the group with age of onset of PD >53. This patient group, treated with antioxidants was compared with another group of early PD patients, treated similarly by another physician except that they did not receive antioxidant therapy. The mean duration of PD before starting on levadopa or a dopamine agonist was 40 months and 24 months respectively.[162] Although this trial was not a controlled trial, it did appear to indicate that the antioxidant treatment could

have potential in delaying the end point of initiation of levodopa to treat the disease, and a large, double-blind trial of antioxidants was seemingly warranted.

Since oxidative stress is an important feature of Alzheimer's disease (AD), the antioxidant capabilities of Vitamin C received attention from those treating AD as well. Low concentrations of antioxidant vitamins C and E have been observed in cerebrospinal fluid (CSF) of AD patients. Kontush et al examined the effect of vitamin C and vitamin E on lipoprotein oxidation in 20 patients with AD.[163] 20 patients were supplemented with either a combination of 400 IU vitamin E and 100mg vitamin C or 400 IU of vitamin E alone. Supplementation with vitamin E and C significantly increased the concentration of both vitamins in plasma and CSF, and the abnormally low concentrations of vitamin C were returned to normal following treatment. Supplementation with both vitamin C and E also significantly decreased the susceptibility of the CSF and plasma lipoproteins to in vitro oxidation, whereas supplementation with vitamin E only was unable to have an effect on lipoprotein oxidizability, even though CSF and plasma concentrations were increased. The investigators explain the superiority of the combined supplementation with vitamins E and C over the supplementation with vitamin E alone by suggesting that ascorbate is a physiologically important co-antioxidant of vitamin E (α-tocopherol). The major mechanism of antioxidant action of α-tocopherol includes inactivation of one free radical by one molecule of α-tocopherol with a subsequent scavenging of a second radical by the α-tocopherol radical formed.[164]

This mechanism is operative under conditions of high oxidative stress. However, the activity of α-tocopherol becomes pro-oxidant under mild oxidative conditions. If no additional free radical interacts with the α-tocopheroxyl radical, it can directly oxidize lipoprotein moieties itself. Therefore, α-tocopheroxyl radicals must be efficiently eliminated from the lipoprotein particle to allow it to develop its antioxidant activity. Elimination of α-tocopheroxyl radical by recycling it directly back into α-tocopherol represents an important mechanism of action of co-antioxidants like ascorbate.[165]

Animal studies of the effect of ascorbate in Huntington's disease have also shown some promise. Mice that express the gene for Huntington's disease show a deficit in striatal ascorbate release during periods of behavioral activity. This deficit was reversed with ascorbate injections, which also improved the behavioral phenotype of repetitive movements.[166,167] This animal work continues, but no clinical trials have been conducted to date.

2.1.3 Coenzyme Q10

Also known as ubiquinone, coenzyme q10 (CoQ10) is an essential cofactor serving as an electron acceptor for mitochondrial complex I. It is also a potent antioxidant in lipid membranes and mitochondria. Studies have shown decreased CoQ10 levels in PD patients that induces alteration in ATP synthesis and damage of the mitochondrial membrane.[168] The first clinical trial was

completed for coenzyme Q10 using dosages of 300, 600, and 1200 mg/day. This placebo controlled, randomized investigation enrolled 80 otherwise untreated PD subjects for 16 months of study drug administration. The primary endpoint was the perceived need for initiation of levodopa, and a secondary endpoint was change in UPDRS score. A positive outcome was found for subjects receiving 1200 mg of coenzyme Q10 per day (but not the 300 and 600 mg/day regimens). This consisted of an improvement of 6.7 points (p = 0.0416) in the adjusted mean score for total UPDRS score. The comparison with placebo treatment was not statistically significant, although a pre-specified endpoint of a positive trend was achieved.[169] Review of the data revealed that most of the benefit observed with the highest dose of coenzyme Q10 was derived from its effect for United Parkinson disease rating scale (UPDRS) Part 2–Activities of Daily Living, rather than to improvement in the motor examination for parkinsonism, UPDRS Part 3–Motor Score. These intriguing results provided great interest in linking the mitochondrial electron chain transfer defect with a possibly correctable mechanism for causation of PD. Because coenzyme Q10 also has antioxidant properties, other actions accounting for its disease-modifying effects are plausible. The authors reporting these findings emphasized that further study of the effects of coenzyme Q10 were warranted before any recommendation as to therapeutic use could be made.[169]

Further investigation of coenzyme Q10 was performed in a NET-PD clinical trial using 2400 mg/day.[23] That dose was one arm of a randomized, placebo-

controlled futility trial linked with investigational compound GPI-1485. Subjects who did not need and were not receiving symptomatic treatment of PD were randomized 1:1:1 among the three treatment assignments (CoQ10, placebo, GPI-1485). The primary outcome measure was the change in total UPDRS score over 12 months from baseline to disability requiring dopaminergic therapy. A number of ratings were conducted in the testing battery in addition to the UPDRS, including measures of depression, cognitive function, quality of life, and disability. Five time points after baseline (1, 3, 6, 9, and 12 months) were used for the testing battery assessing treatment effects. A total of 213 subjects were randomized to the three treatment arms. The mean change in UPDRS score in the group that received coenzyme Q10 was 7.52 (SD: 8.87). Compared with the preset threshold value of 7.46 for progression of PD, found in a previous placebo-controlled study of otherwise untreated parkinsonism (DATATOP), coenzyme Q10 treatment could not be rejected as futile ($p \leq 0.1$). Hence, the study recommended that an additional definitive trial was warranted.[170]

In support of this conclusion, a randomized placebo controlled clinical trial with Coenzyme Q10 in mild PD was conducted in North America. Six hundred participants were randomly assigned to receive placebo, 1200 mg/d of CoQ10, or 2400 mg/d of CoQ10; all participants received 1200 IU/d of vitamin E. Participants were observed for 16 months or until a disability requiring dopaminergic treatment. The prospectively defined primary outcome measure was the change in total UPDRS score (Parts I-III) from baseline to final visit. A

total of 267 participants required treatment (94 received placebo, 87 received 1200 mg/d of CoQ10, and 86 received 2400 mg/d of CoQ10. Treatments were well tolerated with no safety concerns. The study was terminated after a pre-specified futility criterion was reached. At study termination, both active treatment groups showed slight adverse trends relative to placebo. Adjusted mean changes (worsening) in total UPDRS scores from baseline to final visit were 6.9 points (placebo), 7.5 points (1200 mg/d of CoQ10; P = .49 relative to placebo), and 8.0 points (2400 mg/d of CoQ10; P = .21 relative to placebo). Coenzyme Q10 was safe and well tolerated in this population, but showed no evidence of clinical benefit.[171]

2.2. N-acetylcysteine (NAC)

2.2.1 History

NAC was first reported to have clinical benefit in the early 1960's, when it was shown to be an effective mucolytic agent in patients with cystic fibrosis (CF).[172] This discovery arose from the need to deliver reduced sulfhydryl compounds to effect the disruption of disulfide bridges within the glycoprotein matrix of mucous in CF patients. The amino acid residue, L-cysteine was an obvious candidate for such an agent, but unfortunately, it is susceptible to metabolism and undergoes rapid oxidation in solution, generating the inactive disulfide, cystine. Acetylation of the N-terminal of cysteine was found to confer sufficient stability to the molecule to facilitate delivery of reduced sulfhydryl or thiol molecules to work effectively as a mucolytic agent in this clinical setting. .N-acetylcysteine

(Mucomyst) was first approved by the FDA in 1963 as a new molecular entity and was given priority review (NDA **013601).** Mucomyst is a solution for inhalation and is indicated as adjuvant therapy for patients with abnormal, viscid, or inspissated mucous secretions in such conditions as: Chronic bronchopulmonary disease (chronic emphysema, emphysema with bronchitis, chronic asthmatic bronchitis, tuberculosis, bronchiectasis and primary amyloidosis of the lung), acute bronchopulmonary disease (pneumonia, bronchitis, tracheobronchitis), pulmonary complications of cystic fibrosis, tracheostomy care, pulmonary complications associated with surgery, use during anesthesia, post-traumatic chest conditions, atelectasis due to mucous obstruction and diagnostic bronchial studies (bronchograms, bronchospirometry, and bronchial wedge catheterization).[173] Today there are multiple generic manufacturers of Mucomyst.

A new and important role for NAC emerged after subsequent studies investigating its therapeutic potential in acetaminophen poisoning.[174,175] The mechanism of action in this setting was similar to that for CF: delivery of sulfhydryl moieties. However, the mode of action of NAC in acetaminophen overdose was thought to rely not only on the ability of NAC to offer some protection against oxidation, but also through facilitation of rapid membrane permeability due to reduced polarity of the molecule compared to the parent amino acid, cysteine. Cleavage of the acetyl group is thought to reveal free, reduced cysteine, which is then available for incorporation into the highly abundant intracellular antioxidant, glutathione. The benefit in the setting of

acetaminophen overdose is to replenish hepatic GSH that has become depleted through the use of the tripeptide in the drug detoxification process. That NAC remains the treatment of choice more than 50 years after its first use is a testament both to the importance of maintaining cellular GSH reserves and to the exceptional qualities of NAC in helping to replenish this key antioxidant when it becomes depleted. In 2004, the intravenous solution of acetylcysteine became available as Acetadote. This formulation is FDA-approved to be administered intravenously within 8 to 10 hours after ingestion of a potentially hepatotoxic quantity of acetaminophen, and is indicated to prevent or lessen hepatic injury. Since marketing exclusivity for Acetadote expired in 2011, there is now a generic manufacturer, InnoPharma, Inc., which manufactures both the acetylcysteine solution for inhalation and the intravenous formulation.[173]

2.2.2 Physical/Chemical Properties

Acetylcysteine is the N-acetyl derivative of the naturally-occurring amino acid, cysteine. The compound is a white crystalline powder with the molecular formula C5H9NO3S, a molecular weight of 163.2, and chemical name of N-acetyl-L-cysteine. Acetylcysteine has the chemical structure as shown: [173]

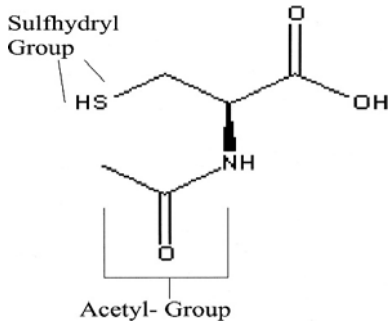


Figure 1. Chemical structure of N-acetylcysteine

2.2.3 Pharmacology of N-acetylcysteine

Over the past several decades, NAC has been studied for numerous and varied potential therapeutic effects. Although the complete mechanisms of its action are as yet unclear, the beneficial effects of NAC have been postulated to include: transmitter effects, redox modulation, neurogenesis, mitochondrial function, immunomodulation and anti-inflammatory activity

NAC modulates several key neurotransmitter systems that are known to be involved in a range of psychopathology, including glutamate and dopamine.[176] Regulation of glutamate synthesis, release, synaptic levels, and recycling is tightly controlled, and dysfunction of this system is implicated in many neuropsychiatric disorders including schizophrenia[177] and addiction.[178] Excessive activation of the N-methyl-D-aspartate (NMDA) glutamate receptor is central to the excitotoxic damage associated with many forms of neuronal damage and degeneration.[179] The cystine/glutamate antiporter, or x(c)-system, is a key element in the control of extracellular glutamate and feedback regulation of

glutamate release.[180] Predominantly expressed in astrocytes in the brain, activity of this transporter is the primary determinant of non-vesicular release of glutamate. The extracellular glutamate activates metabotropic glutamate receptors (mGluR2/3) on presynaptic neurons and regulates vesicular glutamate neurotransmission.[180]

NAC administration can activate the cystine/ glutamate antiporter through provision of additional cystine, thus modulating glutamatergic neurotransmission. Through regulation of this system, NAC has been thought to show beneficial effects in models of schizophrenia, ameliorating behavioral deficits and reversing elevations of extracellular glutamate.[181]

In addition to regulation of glutamate release, NAC, either via GSH or its derivatives, has the capacity to modulate NMDA activity.[182] Early studies suggested that this was through direct binding of the NMDA receptor but more recent reports focus on modulation of redox state by GSH. Given the central role of glutamate signaling in multiple neuropsychiatric disorders it is likely that this activity of NAC is a key component of its therapeutic effect, and explains the rapid increase in clinical studies of NAC in neuropsychiatric disorders in recent years.

Given that dopamine has historically played a lead role in theories underpinning the pathology of many neuropsychiatric disorders (e.g., schizophrenia and

Parkinson's disease), the ability of NAC to modulate dopamine is important. NAC regulation of the cystine/glutamate antiporter and mGluR2/3 as described above can also regulate dopamine release from presynaptic terminals.[180] NAC may also regulate dopamine release via modulation of the redox status of the cell, via the antioxidant effects of GSH and L-cysteine.[183] Dopamine itself is strongly pro-oxidant, forming hydrogen peroxide (H^2O^2) and free radicals through auto-oxidation and normal metabolism, and hence dysregulation of dopamine signaling is thought to be a major contributor to neurotoxicity.[184] Methamphetamine has been shown to evoke strong dopamine release and drive neuronal apoptosis. NAC ameliorates the oxidative stress induced by methamphetamine production and prevents the down regulation of the dopamine transporter elicited by excessive dopamine release.[185] These results not only highlight the therapeutic effects of NAC but also demonstrate the importance of cystine/glutamate transport and GSH regulation of oxidative stress in dopaminergic signaling.[186]

The most-studied aspect of NAC is its role in oxidative homeostasis. GSH levels are efficiently restored by NAC,[187] which acts as a donor of cysteine, the rate-limiting component of GSH synthesis. NAC is also effective in reversing the oxidative stress associated with mitochondrial dysfunction.[188] Approximately 2-5% of oxygen passing through the electron transport system inside the mitochondria results in superoxide. Superoxide is the most well-known of the free radicals as it is commonly produced during the natural pathway of oxidative

phosphorylation.[189] ROS are produced primarily by the mitochondria as a by-product of normal cell metabolism during conversion of molecular oxygen to water. These include superoxide radical, hydrogen peroxide and hydroxyl radical. NAC is an effective free radical scavenger as well as a major contributor to maintenance of cellular GSH status, thereby correcting or preventing GSH depletion.[189] NAC also contributes to the maintenance of oxidative balance through the actions of the cysteine/cystine cycle. Similar to GSH and GSSG, cysteine and cystine are coupled redox partners which help to prevent oxidative cellular dysfunction and injury.[190] Hence, the actions of NAC are multifold and inter-related, with the production of GSH, the cysteine/cystine cycle, and the action of the glutamate/cystine antiporter contributing to maintenance of oxidative balance and cellular function.

Another potential therapeutic avenue for NAC stems from its anti-inflammatory properties. Dysregulation of inflammatory pathways and cytokine levels in both the peripheral and the central nervous system (CNS) are associated with neuropsychiatric disorders. NAC has been shown to reduce IL-6 levels in hemodialysis patients[191] and tumor necrosis factor-a (TNF-a) and IL-1b in patients undergoing surgery.[192] NAC also suppresses production of multiple inflammatory cytokines in burn patients.[193] In rat models of both traumatic brain Injury and focal cerebral ischemia, increased TNF-a and IL-1b levels were reduced following NAC administration.[194,195]

Administration of lipopolysaccharide (LPS), a bacterial endotoxin, induces widespread inflammation and depressive-like symptoms in animal models. However, NAC pretreatment prevents the upregulation of inflammatory cytokines in the brain in response to LPS.[196] Furthermore, sensitization to hypoxic brain injury by LPS and markers of cerebral inflammation are prevented by NAC treatment.[197] The capacity for NAC to reduce neuroinflammation may be through inhibition of the brain inflammatory cells, the microglia. Microglia are brain macrophage-like cells which can be activated by cytokines and in turn produce inflammatory mediators, induce oxidative stress, and promote neurotoxicity.[198] NAC can inhibit activation, cytokine production, and oxidative species production by macrophages and microglia.[199] This effect is likely to be through both stimulation of GSH production and regulation of cystine/glutamate antiporters, regulating oxidative stress and glutamate excitotoxicity.[200]

Overwhelming data support the immuno-modulatory activity of NAC. Clinically, NAC improved the ocular symptoms of subjects with Sjogren's syndrome,[201] enhanced natural killer and T-cell function, and delayed the reduction in CD4+ levels in HIV patients.[202] Administration of NAC to post-menopausal women improved immune functions as exhibited by enhanced phagocytic capacity, leukocytes chemotaxis, natural killer function, and decreased TNF-α and interleukin-8 (IL-8) levels.[203] NAC was also proven beneficial in patients with the autoimmune disorder systematic lupus erythematosus (SLE). In these patients, the mechanism underlying NAC activity was ascribed to a blockade of the

mammalian target of rapamycin (mTOR) in T lymphocytes. Activation of mTOR occurs upon GSH depletion or after exposure to NO, which causes mitochondrial hyperpolarization and can lead to down-regulation of transcription factors and subsequent decline in CD4+, CD25+ T cell population. NAC blocked the activation of mTOR and increased the number of T lymphocytes.[204] Similar in vitro enhancement of T-cell growth and function (production of IL-2) was demonstrated when peripheral blood T cells were treated with NAC.[205]

The molecular mechanisms by which NAC exerts its diverse effects are complex and still largely unclear. NAC has been shown to interact with numerous biochemical pathways. It is very likely that the effects described so far only partially explain the divergent biological effects of NAC, and further studies are required for determining its ability to cross the cell membrane and the blood–brain barrier as well as elucidating its role in cell signaling pathways.

2.2.4 Pharmacokinetics of N-acetylcysteine

Pharmacokinetic parameters of orally and intravenously administered NAC have been determined in several studies of healthy adults,[206,207] adult patients with acetaminophen overdosage,[208] pregnant women, and preterm and near-term infants.[209,210] Pharmacokinetic parameters determined in these studies are summarized in Table 1. The results of these studies have varied depending on the analytical methods used, dose, formulation, sample collection timing and route of administration.

Table 1. Published NAC pharmacokinetic parameters

Study	**Dose**	**Form**	**Mean $t_{1/2}$ (h)**	**Mean CL (L/h/kg)**	**Mean Vss L/kg**
Borgstrom et al 1986[206] 10 adult healthy volunteers	600 mg iv 600mg oral	Non-protein-bound NAC	2.27 iv (elimination)	0.211	0.33
Olsson et al, 1988[207]	200mg iv 400 mg oral*	Total NAC	5.58 iv (terminal)	0.11	0.47

6 healthy volunteers					
Prescott et al1989[208] 17 patients with Acetaminophen overdose	150mg/kg over 15 mins followed by 50mg/kg in 4 h, and 100mg/kg over 16h	Total NAC	5.7 (±2.9) (terminal)	0.19	0.54
Ahola et al, 1999[210] 10 preterm infants	4.2mg/kg/h for 24 h (continuous infusion)	Total NAC	11 (elimination)	0.037	.57
Weist et al. 2014[209] 11 pregnant women, 5 preterm infants,	100mg/kg iv q4h 12.5mg/kg q12h	Total NAC	1.2 7.5	0.26 0.045	0.41 0.47

6 near term infants	25mg/kg q12h		5.1	0.07	0.34

* Oral bioavailability of total NAC: 9.1%

2.2.5 Clinical use of N-acetylcysteine

Although the FDA-approved indications for the use of NAC are as a mucolytic and as an antidote for acetaminophen overdose, NAC has been studied in a wide variety of therapeutic areas for multiple indications over the last several decades. A recent search of NAC on clinicaltrials.gov, showed 286 clinical trials in varying stages of completion. The subject of these studies continues to be quite varied; Smoking cessation, alcohol dependence, pathological gambling, sickle cell disease, renal failure, intra-amniotic infection, autism, self-injurious behavior, bipolar disorder, polycystic ovary disease, cognitive dysfunction, schizophrenia, neurotoxicity of chemotherapy, pulmonary fibrosis, neuronal ceroid lipofuscinosis, metabolic disorders, opioid abstinence, and fingernail biting are just a short list of the areas being studied with NAC either alone or as adjunct therapy.[211] In recent years, the role of NAC in the treatment of psychiatric and neurologic disorders has attracted the attention of researchers as well as clinicians. In the following chapters, the focus will turn to the role of NAC in two neurodegenerative disorders in which NAC may play an important role.

Chapter 3

Pharmacology of Intravenous N-acetylcysteine with Hematopoietic Cell Transplantation in Pediatric Inherited Metabolic Disorders

3.1 Background

Since the 1980s, hematopoietic cell transplantation (HCT) has been used as a treatment for metabolic disorders. For lysosomal disorders, stable engraftment following transplantation has the potential to provide a source of a necessary enzyme for the life of the patient. Recombinant enzyme replacement therapy is available for those disorders that do not have a primary neurologic component. However for diseases affecting the CNS, intravenous enzyme is ineffective due to its inability to cross the blood-brain-barrier. For disorders such as adrenoleukodystrophy, specifically the cerebral form of ALD, HCT remains the only effective form of treatment. In ALD, however, the phenotype and extent of disease at the time of transplantation are of fundamental importance in determining outcomes after transplant. In 2004, Peters et al reported on the international HCT experience and showed that pre-HCT magnetic resonance imaging (MRI) severity scores and measures of disability, such as the ALD-Disability Rating Scale (ALD-DRS), developed at the University of Minnesota, showed marked differences in outcomes after transplant.[212] Boys with advanced disease, and therefore higher MRI (Loes) scores and higher disability rating scores had worse outcomes after HCT. As a result of this data from the international experience, HCT was subsequently generally reserved for early stage cerebral ALD. Meanwhile, the University of Minnesota's Pediatric Blood and Marrow Transplantation (BMT) Program had developed a national, if not international reputation as being a leader in HCT for metabolic and other disorders. Therefore, boys with a new diagnosis of ALD were often referred to

the University of Minnesota's program, often after having a delayed diagnosis, and therefore often presented with advanced disease. Physicians at the University of Minnesota's BMT program were faced with having no alternative treatment for those patients referred to them with late-stage ALD.

Based on the work of Powers et al, who showed that oxidative stress played a key role in ALD as assessed by standard biochemical and immunohistochemical markers in the ALD-mouse model as well as ALD patients,[213] physicians turned to NAC as a potential adjunctive therapy with HCT in late-stage ALD. The choice of NAC was based on the premise that NAC was a well-known, generally safe antioxidant with established, safe dosing for children. The initial study included three boys approximately 10 years old with a confirmed diagnosis of ALD and Loes scores of > 14 who were administered an initial NAC 140mg/kg intravenous infusion followed by 70mg/kg four times per day either orally or iv, per an approved BMT protocol. All three of the boys treated with adjunctive NAC therapy were surviving at 8-11 months post BMT, compared to zero of 8 boys, also with severe ALD, who were previously treated with HCT alone.[214] The difference in survival rates at 12 months post-transplant between these groups of young boys, both with advanced ALD, spurred clinician/researchers at the University's BMT program to investigate the use of NAC in ALD.

The Center for Orphan Drug Research at the University of Minnesota (Dr. James Cloyd) was contacted by the pediatric neurologist (Dr. Laurence Charnas) who

was involved in the NAC study. After a short afternoon discussion in which the data from the small, 3-patient NAC study were discussed, it was decided that a larger study of the pharmacology of NAC was necessary. Thus began my involvement with the study of NAC in ALD and other metabolic disorders with neurologic effects.

3.2 My Role in This Study

- Assisted in design of the pharmacokinetic studies, based on review of existing literature on PK of NAC
- Assisted in incorporation of PK elements into existing BMT protocols for metabolic disorders (both early stage and late-stage disease protocols)
- Co-wrote grants for supplemental funding
    - NIH/ NICHD R21 (PAR 06-342: Innovative Therapies & Clinical Studies for Screenable Disorders)- not funded
    - AHC- University of MN- funded
- Collaborated with study nurses to incorporate PK protocol into BMT protocol for floor nurses
- Training of BMT floor nurses during all shifts regarding the protocol and need for timing and handling of blood samples in the clinical setting, forms, etc.
- Timely pick up and transport of all blood samples from BMT floor to CODR

- Initial sample preparation
- Blood sample analysis (with CODR scientist)
- PK and safety data analysis
- Writing of draft manuscript (not yet published)

3.3 Introduction and Study Objectives

The inherited metabolic disorders included in this study are lysosomal storage diseases (LSD) and ALD. The inherited lysosomal storage diseases included are mucopolysaccharidosis Type I (MPS 1, Hurler's Syndrome), MPS VI (Maroteaux-Lamy Syndrome). These LSDs involve a deficit in the lysosomal enzymes, alpha-L-iduronidase and aryl sulfatase-B, respectively, resulting in accumulation of glycosaminoglycans, increase in oxidative stress, and resultant damage to organs, including heart, lung, liver spleen and central nervous system.[215] Mucolipidosis I (Sialidosis) is another lysosomal storage disease in which the enzyme neuraminidase is deficient, resulting in accumulation of sialic acid and damage to the CNS.[216] Based on the premise that the antioxidant properties of NAC could be helpful in all of these inherited metabolic disorders,[217] the NAC pharmacokinetic (PK) study protocol was integrated into the existing BMT study protocol for these patients.

While NAC may have clinical utility as an adjunctive therapy in LSDs and ALD, in order to develop clinical trials to assess efficacy and to optimize dosing regimens, pharmacokinetic studies in children, and in these specific populations

are required to answer the following questions: 1.) How do the pharmacokinetic parameters of iv NAC in these patients differ from that of healthy adults and pre-term newborns? 2.) Does the preparatory regimen for HCT alter these PK parameters? 3.) How does the HCT itself alter the PK parameters? 4) Does NAC infusion result in increased plasma and red blood cell concentrations of glutathione? The objective of this study was to determine PK parameters and examine the pharmacology of NAC in these patients, in an attempt to answer these important questions.

3.4 Methods

The study was approved by the University of Minnesota Human Research Protection Program and was listed on clinicaltrials.gov: Standard risk protocol: NCT 00668564, High risk protocol: NCT 01626092

Subjects

Written informed consent was obtained for all study participants prior to enrollment. A total of 20 children were enrolled in the study: 18 males, 2 females, ages 2-15 years. Participants were diagnosed with ALD (n=17) MPS I (n=1), MPS VI (n=1) or Mucolipidosis I (n=1) prior to enrollment based on plasma VLCFA, enzyme assays or cell cultures. Subject characteristics and blood samples analyzed are shown in Table 2.

Table 2. Subject characteristics and blood samples analyzed

Subject Number	Diagnosis	NAC Dose (mg)	*Plasma*			*Red Blood Cells*		
			AUC 1	AUC 2	AUC 3	AUC 1	AUC 2	AUC 3
1	MPS VI	1650	x	x	x			
2	ALD	2800	x	x	x			
3	ALD	1750	x	x	x			
4	ALD	2000	x	x	x			
5	ALD	2000	x	x	x			
6	ALD	1700	x	x	x			
7	Mucolipidosis I	4000	x	x		x	x	
8	ALD	1800	x	x				
9	ALD	1700	x	x	x			
10	ALD	1400	x	x		x	x	
11	ALD	1540	x	x	x			

12	ALD	1400					x	x
13	ALD	2000		x	x		x	x
14	ALD	1400		x	x		x	x
15	ALD	3000		x	x			
16	ALD	2500		x	x			
17	ALD	2000		x	x		x	x
18	ALD	2300		x	x		x	x
19	ALD	3000		x	x		x	x
20	MPS I	1000		x	x			

Study Protocols

Based on disease progression and assessment of post-transplant morbidity and mortality risk, participants were assigned to one of two study protocols. High risk participants (n=12) were enrolled in the high risk, reduced intensity protocol, with HCT occurring on day 0. Subjects were admitted 12 days prior to HCT. The HCT preparatory regimen consisted of Campath-1H on day-12 through Day-8, continuous infusion of ondansetron beginning on day-7, clofarabine on day-7 through day-3, Cyclosporin A therapy beginning on day-3 through day+100, and a single infusion of melphalan on day-2. Total body irradiation dose was given in

a single fraction on day -1. Post-transplant immune suppression included mycophenylate mofetil (MMF) day -3 through day 30, and cyclosporine (CSA), day -3 through day 100. NAC was administered from day -12 through day 100.

A standard risk protocol was used for those participants with less advanced, lower-risk disease (N=8). The preparatory regimen consisted of the following: Campath-IH on days -21 through -19, cyclophosphamide, days -9 through -6, busulfan, days -5 through -2. Day -1 was a rest day with no treatment. HCT occurred on Day 0. Post-transplant immune suppression included MMF day -3 through day +42, CsA day -3 through day 100-180 (depending on the source of donor cells). NAC was administered from day +1 through day +100.

In both protocols, intravenous NAC, 70mg/kg was infused over one hour every 6 hours until the participant was discharged from the hospital. After discharge, administration intervals were adjusted to three times daily using the same dose, and were administered by home health nurses.

Blood samples were collected on Day-7 (AUC 1) at 0.25,0.5,1,2,3,5 and 8 hrs post infusion, Day+7, (AUC 2) at 0.25, 0.5,1,2,3 and 5 hrs post infusion, and Day+21 (AUC 3) at 0.25, 0.5 ,1,2,3 and 5 hrs post infusion, as noted in Table 2. Red blood cells and plasma were separated and frozen at -20^{o}C until analysis.

Sample Analysis

Plasma and red blood cell samples were analyzed by electrospray ionization (ESI) using a Hewlett-Packard 1100 series high performance liquid chromatographer with a quadropole mass spectrometer (HPLC/MS) (Agilent Technologies, Santa Clara, CA) and a validated assay consisting of quality control (QC) standards, calibration standards, and internal standards. Briefly, analytes were separated using a Zorbax Eclipse (Agilent Technologies) XDB C18 column (150 x 3.0 mm, 3.5µ particle size) with a mobile phase consisting of 20 mM ammonium formate buffer (pH 3.5) and acetonitrile (Sigma; 98:2 vol/vol). Analytes were detected in negative ion mode with quantitation ions at m/z 120, m/z 162 and m/z 306 for Cys, NAC, and GSH, respectively. The deuterated forms of Cys, NAC, and GSH were used as internal standards and quantified at m/z 122, m/z 165, and m/z 309, respectively. The flow rate was 0.35 mL/min, and the run time was 10 min.

Analysis of plasma samples

Plasma QC standards (n=3) were prepared prior to analysis using diluted plasma (1:1 with 20mM ammonium formate buffer) and solubilized Cys, NAC, and GSH in buffer (range 48-12 µg/mL, 240-640 µg/mL, and 24-80 µg/mL, respectively). QC standards were frozen at -20^{o}C until day of analysis. Calibration standards consisted of six mixed standards (range of 0-40 µg/mL), two high-concentration NAC standards (80 µg/mL and 160 µg/mL), and zero. Volumes for sample processing were as follows: 250 µL plasma (subject, QC, or diluted), 50 µL

internal standard, 100 µL 0.05 mg/mL dithioerythritol (DTE), 100 µL buffer, and 50 µL calibration standard (if appropriate). The final internal standard concentration was 20 µg/mL for deuterated GSH and Cys and 120 µg/mL for deuterated NAC. A “blank” plasma sample containing only 250 µL diluted plasma was included in assay preparation for subtraction of background. All calibration standards were analyzed in triplicate. QC standards were analyzed in quadruplicate.

All samples were incubated in a water bath for 30 min at 37^{o}C and 85 rpm. Following incubation, 2 mL of methanol was added to each tube to precipitate the protein. All samples were centrifuged at 2000 rpm for 10 min. The organic layer was conserved and evaporated at 37^{o}C by gaseous nitrogen (15 psi) using a TurboVap® LV Concentration Evaporator. Sample contents were reconstituted in 300 µL of buffer and filtered through Acrodisc® nylon syringe filters into HPLC vials for analysis.

Analysis of red blood cell samples

Red blood cell QC and calibration standards were prepared similarly to plasma standards except diluted blood was used as the medium. The QC concentration range was 6-50 µg/mL for Cys and NAC (n=3) and 125-450 µg/mL for GSH (n=3). The final calibration standard range was 0-100 µg/mL for Cys and NAC and 0-500 µg/mL for GSH. Calibration standards were comprised of six mixed compound standards, one high NAC standard (500 µg/mL), and zero. Volumes

for sample processing were as follows: 50 µL blood (subject, QC, or diluted), 25 µL internal standard, 50 µL red blood cell lysis buffer, 50 µL DTE, and 25 µL calibration standard (if appropriate). HPLC grade water (25 µL) was also added to QC and patient samples. The final internal standard concentration was 100 µg/mL for GSH, Cys, and NAC. A “blank” blood sample (no internal or calibration standard) was included in assay preparation for subtraction of background. Further blood analysis mimicked that of the plasma samples described above.

Data Analysis

Calibration curves were constructed for each analyte in plasma and blood using peak area response ratios of analyte to internal standard and known concentration. Concentrations of Cys, NAC, and GSH were calculated from the appropriate calibration curve. The lower limit of quantitation was 5 µg/mL for NAC and Cys and 50 µg/mL for GSH.

Pharmacokinetic Data Analysis

Pharmacokinetic analysis of intravenous NAC was completed with Phoenix WinNonlin 6.2 (Pharsight Corporation, Mountain View, CA, USA). Non-compartmental analysis was used to calculate area under the curve. Time to maximum concentration (Tmax), minimum concentration (Cmin), maximum concentration (Cmax), volume of distribution, clearance and half-life were also determined.

For each PK parameter, the data were analyzed using repeated measures analysis of variance (ANOVA) to determine significant differences based on dosing period (AUC 1 vs 2 vs 3). Significance was determined at the $p < 0.05$ level**.** Accuracy and precision data were determined for quality control samples for both assays and are reported in table 3.

Table 3. Accuracy and precision data for plasma and red blood cell assays

Assay	**Nominal conc. (µg/ml)**	**NAC(N=20)**		**CYS(N=20)**		**GSH (N=20)**	
		A* (%)	**P** (%CV)**	**A* (%)**	**P** (%CV)**	**A* (%)**	**P** (%CV)**
Plasma	LQC	98.34	9.05	131.73	20.98	86.56	13.0
	MQC	88.72	5.69	85.24	14.98	88.66	9.51
	HQC	90.62	6.12	88.16	8.79	91.21	6.42
RBCs	LQC	106.64	17.9	106.71	23.7	107.41	12
	MQC	95.38	9.8	108.50	24.7	97.45	11.1
	HQC	108.97	10.6	95.47	12.9	95.47	13.3

A*= accuracy, P= precision**

3.5 Results

The NAC concentration profiles in plasma and red blood cells following 70 mg/kg NAC infusion are shown in Figure 2. The NAC pharmacokinetic parameters are reported in Table 4.

a. **Plasma** **b. Red Blood Cells**

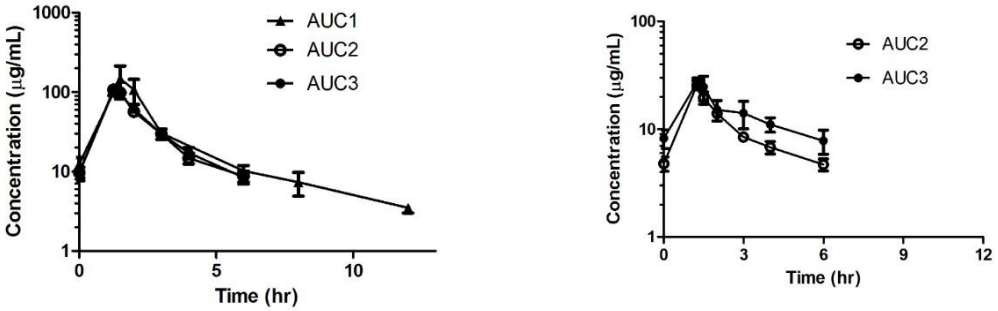


Figure 2. NAC concentrations in plasma (a) and red blood cells (b) following NAC infusion (70 mg/kg). Values are expressed as mean ± standard error of the mean (SEM).

Table 4. Pharmacokinetic parameter values for NAC following 70 mg/kg NAC infusion.

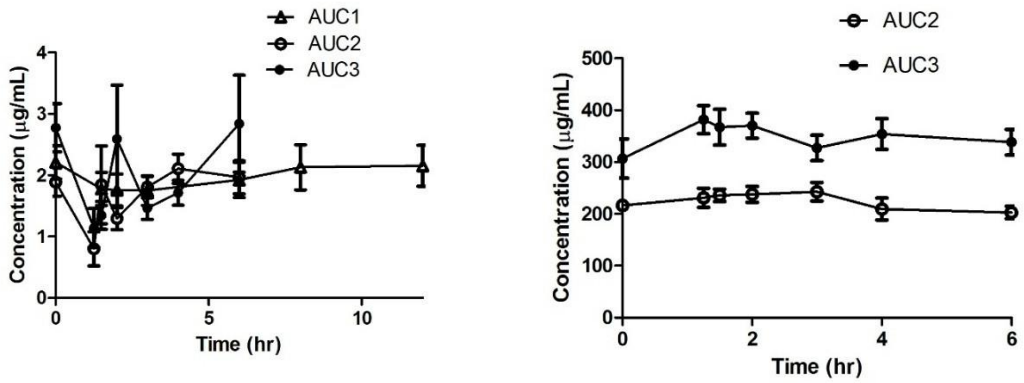
	Parameter	AUC 1	AUC 2	AUC 3
		Mean (SD), *% CV*	**Mean** (SD), *% CV*	**Mean** (SD), *% CV*
Plasma	**C_{min}** (µg/mL)	**11** (6), *56*	**8** (5), *64*	**9** (8), *95*
	C_{max} (µg/mL)	**157** (189), *120*	**117** (41), *35*	**106** (28), *27*
	$AUC_{0\text{-}6hr}$ (hr*µg/mL)	**313** (266), *85*	**222** (92), *41*	**225** (82), *37*
	$AUC_{0\text{-}inf}$ (hr*µg/mL)	**356** (306), *86*	**248** (105), *43*	**249** (92), *37*
	$t_{1/2}$ (hr)	**1.5** (0.5), *31*	**1.7** (1.1), *67*	**1.5** (0.5), *30*
	CL_{ss} (L/hr/kg)	**0.3** (0.15), *49*	**0.4** (0.2), *50*	**0.4** (0.2), *50*
	V_{ss} (L/kg)	**0.81** (0.55), *67*	**0.80** (0.41), *52*	**0.80** (0.30), *38*

	V_z (L/kg)	**0.77** (0.53), *69*	**0.88** (0.61), *69*	**0.77** (0.40), *51*
Red Blood Cells	**C_{min}** (µg/mL)	**2.2** *	**4.4** (2.7), *61*	**7.0** (4.4), *63*
	C_{max} (µg/mL)	**18.6** *	**27** (4.9), *18*	**35** (12), *36*
	$AUC_{0\text{-}6hr}$ (hr*µg/mL)	**33** *	**61** (21), *35*	**78** (51), *65*
	$AUC_{0\text{-}inf}$ (hr*µg/mL)	**40** *	**71** (26), *37*	**108** (80), *74*
	$t_{1/2}$ (hr)	**1.4** *	**2.6** (1.0), *39*	**2.2** (1.5), *68*
	CL_{ss} (L/hr/kg)	**0.06** *	**0.05** (0.02), *32*	**0.04** (0.02), *55*
	V_{ss} (L/kg)	**0.14** *	**0.16** (0.05), *31*	**0.10** (0.02), *20*
	V_z (L/kg)	**0.14** *	**0.17** (0.05), *31*	**0.04** (0.02), *55*

NAC reached maximum plasma concentrations of approximately 157 ug/mL at 1.5-1.7 hours post start of infusion and maximum red blood cell concentrations of 35 ug/mL. Red blood cell NAC concentrations ranged from 12-33 % of plasma NAC concentrations. Total red blood cell exposure based on $AUC_{0\text{-}inf}$ was 11%, 29% and 43 % of total plasma exposure for AUC 1, AUC2 and AUC3 respectively. Half- life of NAC was approximately 1.5 hours and was similar in plasma and red blood cells.

Mean glutathione concentrations are shown in figure 3. Mean plasma glutathione concentrations initially decreased from baseline following NAC infusion. Concentrations then rose to at least baseline levels several hours after NAC infusion. Red blood cell concentrations of glutathione increased slightly after

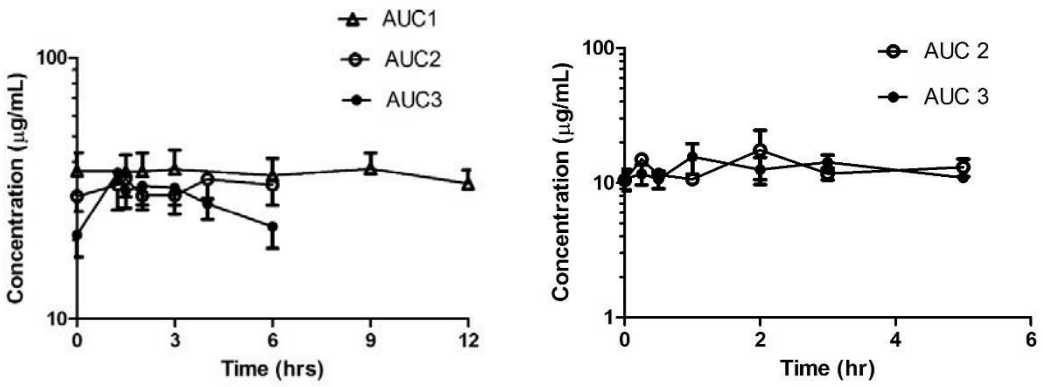
NAC infusion and then stabilized. RBC concentrations were approximately 100 times that of plasma concentrations, as expected. Increased variability of glutathione concentrations was noted, particularly in plasma. Although no overall difference was seen in plasma concentrations over AUC 1, AUC 2 and AUC 3, an increase in RBC concentrations was observed in AUC 3 versus AUC2.



a. **Plasma** b. **Red blood cells**

Figure 3. Glutathione concentrations in plasma (a) and red blood cells (b) following NAC infusion (70 mg/kg). Values are expressed as mean ± standard error of the mean (SEM)

Cysteine concentration-time profiles are reported in Figure 4. Mean cysteine concentrations remained relatively stable after NAC infusion in both plasma and red blood cells. An increase in plasma cysteine concentrations was seen in AUC 3, but plasma concentrations returned to baseline after 6 hours.



a. Plasma **b. Red blood cells**

Figure 4. Cysteine concentrations in plasma (a) and red blood cells (b) following NAC infusion (70 mg/kg). Values are expressed as mean ± standard error of the mean (SEM)

No significant differences were found between AUC1, AUC2 and AUC3 in the PK parameters AUC, Cmax, half-life, clearance or volume of distribution.

Adverse events experienced by the subjects were nausea and vomiting, and were considered mild to moderate in severity. With the multiple HCT-related medications being administered, it is difficult to determine causality of these adverse events. No serious adverse events were reported during the study and no subject discontinued NAC due to adverse events. There were no allergic or anaphylactoid reactions reported during the study.

3.6 Discussion

This is the first report of NAC pharmacokinetics in the pediatric population undergoing HCT. We report an average half-life of 1.5-1.7 hours ±1.1 hr, which is similar to that reported in the literature for adults,[206,209] but substantially shorter than the half-life of 11 hours reported in pre-term newborn infants.[210] AUC_{0-6} and AUC_{0-12hr} were calculated using the concentration data from AUC 1. The AUC_{0-6} was approximately 95% of $AUC_{0-12.}$ Clearance in these subjects was similar to that of pregnant women,[209] slightly higher than adult men,[206] and 90-150% higher than that of preterm newborn infants.[209,210] Exposure as measured by AUC_{0-6hr} did not appear to be affected by the pre and post- transplant regimen, and remained consistent over AUC 1, AUC 2 and AUC 3, before and after HCT.

Although no overall difference was seen in plasma glutathione concentrations over AUC 1, AUC 2 and AUC 3, an increase in RBC concentrations was observed in AUC 3 versus AUC2. This increase may be related to the effects of NAC and the provision of cysteine for glutathione synthesis. It may also be the effect of HCT itself or recovery from the effects of the peri-transplant regimen.

Based on these data, NAC dosing does not appear to result in increased cysteine. This lack of effect on cysteine concentrations has been reported by others.[218] However, there may be some effect of NAC on levels of reduced versus oxidized cysteine concentrations or unbound versus bound cysteine, therefore increasing the “therapeutically available” concentrations. Likewise with

glutathione, NAC may be increasing the availability of reduced glutathione and therefore exerting its therapeutic effect. A shortcoming of this study was that total cysteine and glutathione were measured. Future studies that measure both the oxidized and reduced forms of cysteine and glutathione may delineate additional information on the therapeutic effect of NAC.

AUC calculations based on 6 hour concentration data was 95% of the AUC based on 12 hr data. We therefore report a half-life of 1.5 hrs based on concentrations to 6 hours which we believe represents the effective half-life of NAC.

The lack of adverse events experienced by subjects in this study is striking. Allergic and anaphyactoid type reactions to N-acetylcysteine are fairly common among patients being treated with NAC for acetaminophen overdose.[219] Although the number of subjects in this study was relatively small, we would have expected to see at least a few subjects experience an anaphylactoid type adverse event. One potential reason for the lack of these adverse events is the fact that the subjects were on immunosuppressants as part of the peri-transplant regimen. Overall, NAC appears to be safe and well tolerated in this population.

The data from this pharmacokinetic study in children has implications for future studies of NAC in pediatric populations undergoing HCT. Pharmacokinetic parameters as measured in this study appear to be similar to published data on

adults, and remain largely unaffected by the transplant preparatory regimen used in this study, or by the transplant itself. Although total cysteine did not appear to increase in either plasma or red blood cells as a result of NAC, future studies will require measurement of both reduced and oxidized cysteine, to ascertain if there is indeed an increase in the reduced, and therefore “available” form of cysteine. Likewise, reduced and total glutathione measurements will be helpful in determining if the GSH redox ratio is altered as a result of NAC administration.

The data from this PK study can be used to guide NAC dosing for additional work in this pediatric population to determine the blood and brain effects of NAC in those with metabolic disorders with neurologic effects.

Chapter 4

Effect of N-acetylcysteine on Brain Glutathione Concentrations in Gaucher and Parkinson Disease Patients

4.1 Rationale for the study of NAC in PD and GD

Although modest increases in intracellular GSH were detected in red blood cells after repeated i.v. dosing of NAC in children with metabolic disorders, the effect of NAC on brain GSH concentrations remained unknown. Since the effect of NAC on neurodegenerative disorders involving oxidative stress is reliant on the ability to cross the blood-brain-barrier, the next study of NAC would logically be the study of brain effects. Fortunately, modern magnetic resonance spectroscopy (MRS) methods allow for the quantification of a number of metabolites in the brain, including GSH.[220] Although MRS methods have been used to assess cerebral lesions in boys with ALD in the past,[221] a prolonged, PK/ MRS study would not be feasible in this vulnerable population.

Since GSH depletion in PD and an impaired adaptive response to oxidative stress in GD have both been observed, antioxidants that increase GSH may have a protective effect in these disorders. Unfortunately, past PD research using intravenously administered GSH failed to provide a definitive benefit.[222] This failure may be related to a suboptimal dosing regimen, but more likely is due to the saturable mechanism for GSH passage across the blood-brain barrier as well as a lack of uptake into neurons.[223]

A possible way to boost CNS GSH levels while avoiding the above is to employ NAC, a membrane-permeable cysteine precursor which serves as the rate limiting substrate for GSH biosynthesis. NAC increases intracellular GSH in

human erythrocytes,[218] however its effect on human brain GSH concentrations is unknown. Despite in vivo studies of NAC in animal models of neurodegenerative disease,[224,225] and a small clinical trial of oral NAC in patients with late stage Alzheimer's disease,[226] it has yet to be shown that NAC can in fact alter GSH concentrations in the human brain. We hypothesized that intravenously administered NAC increases brain GSH concentrations and blood GSH/GSSG ratios. We utilized ultra-high field MRS methodology[227] to measure the effect of NAC administration on cerebral GSH levels and mass spectrometry to measure its effects on blood redox status.

4.2 My Role in the Study of Brain Effects of NAC

- Co-wrote 4 grant applications:
    - Minnesota Medical Foundation- initial funding
    - Minnesota Futures Grant- not funded
    - Michael J. Fox Foundation: Repositioning Drugs for Parkinson Disease- not funded
    - LDN/NIH Rare Disease Clinical Research Network (RDCRN) - funded
- Designed study with study team
- Wrote study protocol
- IRB application, submission
- IND application/ Request for waiver
- Re-wrote protocol with NIH input

- Re-submitted IRB application with changes from NIH
- Developed all case report forms and study manual
- Developed regulatory binder with study expert from Neurology department
- Assisted in development of NIH- compliant patient database
- Study coordinator responsibilities:
    - Filed all paperwork with CTSI for nursing staff, trained nursing staff
    - Recruited, screened, enrolled, scheduled subjects, filed necessary paperwork for reimbursement of study participants
    - Prepared study binders for each subject, delivered drug order to investigational pharmacy
    - Day of study coordination:
        - Coordinated study staff, nurses, CMRR staff, materials, equipment, exam rooms, MRI suite, pick up and transport of study drug
        - Met subjects, assisted with consent, baseline assessment
        - Administered NAC infusion, coordinated

blood sample handling and transport to lab, timed study

- Filed all IRB, NIH/RDCRN paperwork
- Analyzed PK and safety data
- Coordinated Study Audit by NIH/ RDCRN, May 29-30,2013

4.3 Study Methods

Subjects

This study enrolled 3 people with PD, 3 people with GD, and 3 healthy controls (HC). The study protocol was approved by the University of Minnesota Human Research Protection Program, the National Institutes of Neurological Disorders and Stroke (Protocol #6721), and was listed on ClinicalTrials.gov: NCT01427517. All participants gave written informed consent before enrollment. Non-demented individuals with mild to moderate PD (Hoehn and Yahr stage 2) were recruited from the University of Minnesota's Movement Disorders Center. Patients with genetically and metabolically confirmed GD type 1 were recruited from the University of Minnesota and the Lysosomal Disease Network/NIH. The study included adult male (N=4) and female subjects (N=5), age (years), gender and disease classification: PD: 52F, 53F, 58F; GD: 18M, 50M, 64M; and HC: 58F, 58M, 58F. All subjects were on stable treatment regimens for 1 month prior to enrollment. Use of

antioxidant supplements, including coenzyme Q-10 and vitamin E, was not allowed within 3 weeks of enrollment. Individuals with a history of asthma or bronchospasm were excluded from the study.

Clinical Assessments & Study Scheme

Age, ethnicity, gender, disease duration, weight, smoking status, use of alcohol and illicit drugs, Unified Parkinson's Disease Rating Scale score (I-III), Hoehn & Yahr staging, Montreal Cognitive Assessment Score, medications, supplements and vital signs were recorded at baseline. Following collection of a baseline blood sample, the subject was placed into the MR scanner to determine the baseline brain GSH concentration. The subject was then removed from the scanner and NAC (150 mg/kg) was administered intravenously over 1 hour. The subject was placed back into the scanner ~30min following the start of infusion and brain GSH measurements and blood samples were collected at 15 minute intervals until 1 hour after the end of the infusion.

MRS Protocol

Brain MR scans were performed using a 7T, 90-cm horizontal bore magnet (Siemens MAGNETOM) and a quadrature surface coil, as described previously.[219] Images acquired with a 2 x 1 x 2 mm

resolution MPRAGE sequence were used for the selection of the volume-of-interest (VOI). All first- and second-order shims were adjusted using FASTMAP with echo-planar imaging readout.[228] Spectra were acquired from the occipital cortex (22 x 22 x 22 mm^3) with a modified semi-LASER sequence (echo time TE = 26 ms, repetition time TR = 5 s).[229] Unsuppressed water spectra acquired from the same VOI were used to remove residual eddy current effects and as a quantification reference. Single-shot data were saved during acquisition; individual FIDs were frequency and phase corrected prior to summation.

Metabolite Quantification

Metabolites were quantified using LCModel[230] as described before.[227,220] The metabolite model spectra were generated based on previously reported chemical shifts and coupling constants.[231,232] Macromolecule spectra were acquired from the occipital cortex using an inversion recovery sequence (TR = 2 s, inversion time TI = 0.680 s).[233] Metabolites quantified with Cramér-Rao lower bounds (CRLB, estimated error of the metabolite quantification) > 50% or correlation coefficient < -0.5 were classified as not detected.[234] Concentrations were not corrected for T_1 and T_2 effects or cerebrospinal fluid contribution to the VOI.

GSH/GSSG Redox Ratio

Sample Collection and Analysis

Blood samples were collected via a catheter that was placed in the subject's lower leg into a tube containing EDTA. To prevent GSH oxidation, 1 mL of whole blood was immediately mixed with 0.5 ml of 3% meta-phosphoric acid (Sigma). The blood samples were stored at 4°C and analyzed the same day as blood collection. Fifty microliters of blood were extracted with 2 mL of methanol (Fisher). The supernatant was evaporated and reconstituted in 300 µL of mobile phase and filtered into high performance liquid chromatography vials using Acrodisc® nylon syringe filters. The chromatographic system used consisted of a Hewlett-Packard 1100 series (Agilent Technologies) with a quadrupole mass spectrometer (model G1946A). The analytes were separated using a Zorbax Eclipse XDB C18 column (150 mm x 3.0 mm, 3.5µ particle size) and eluted isocratically with a mobile phase consisting of 20 mM ammonium formate (Fluka) buffer (pH 3.5) and acetonitrile (Sigma) (98:2, v/v). The analytes were detected using an electrospray ionization-MS system in the positive ion mode (quantitation ions: m/z 122, m/z 164, m/z 308, and m/z 613 for cysteine, NAC, GSH, and oxidized glutathione, respectively). Each sample was analyzed in triplicate. The GSH/GSSG redox ratio was determined using the peak area response ratio for GSH and GSSG.

GSH/GSSG redox ratio was reported as the mean of the triplicate values with all values within 50 percent of the mean.

Data Analysis

Percent change from baseline was calculated for both blood GSH/GSSG ratios and brain GSH levels for each subject at each time point. Maximal percent change for each subject was determined.

4.4 Results

Blood GSH/GSSG ratios increased following the start of NAC infusion, reaching a maximum at approximately 60-75 minutes. Brain [GSH] also increased with maximal values observed at approximately 90-110 minutes after the start of infusion. Subjects with the greatest percent change in blood GSH/GSSG ratio after NAC infusion also had the greatest percent change in brain [GSH], with the exception of subject PD03. (Figure 1) The average maximal percent change from baseline in brain [GSH] was 55%, 41% and 34% in the PD, GD and HC groups, respectively. Although brain [GSH] reached maximal percent change from baseline at 90-110 minutes and then began to decline in all subjects, none of the subjects had returned to their baseline brain [GSH] at 120 minutes post NAC infusion.

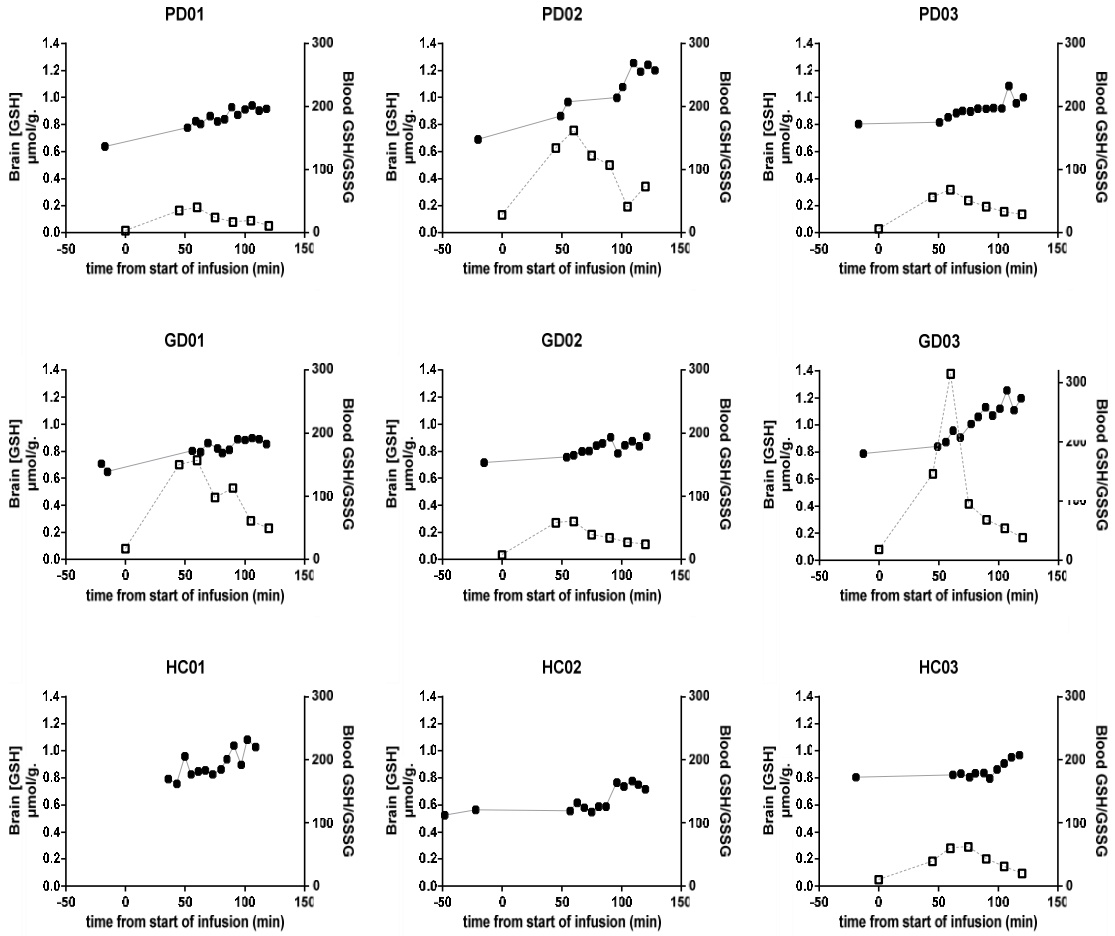


Figure 5. Blood GSH redox ratio (open squares) and brain GSH concentrations (closed circles) in PD, GD and healthy control subjects. Blood GSH redox ratios for HC 01 and HC02 not shown, due to difficulty with GSH/GSSG stabilization prior to bioanalysis.

4.5 Discussion

In this proof-of-concept study, we show that brain GSH concentrations and blood GSH redox ratios increase following intravenous NAC administration and thereby demonstrate the feasibility of altering and

monitoring changes in peripheral and central antioxidant status. These results support the hypothesis that NAC enhances GSH synthesis in the brain. While the ultimate question as to whether altering brain [GSH] will affect the course of PD or GD remains unanswered, this represents the first demonstration that MRS can measure pharmacodynamic changes in response to intravenous NAC administration in PD, GD and controls. The [GSH] measured by MRS in the occipital cortex VOI also includes the contribution of blood GSH. However, blood makes up only ~3 percent of the volume in brain.[235] Thus, the change detected in brain [GSH] in this study could not be due primarily to the contribution of blood GSH because the mean maximal increase in blood GSH of 30% measured in this study would only lead to a MRS voxel [GSH] increase of 0.9%. In addition, the maximal change in the brain [GSH] occurred with a ~30 min delay relative to the redox change in blood (Figure.1), further substantiating the limited effect of blood on the brain measurement.

Although the average maximal percent change from baseline in brain [GSH] appeared to differ among the subject groups, the number of subjects in this study is too small to draw any definitive conclusions. A study involving a larger number of subjects is required to determine if individuals with PD or GD have altered baseline GSH levels that may be due to their subtype or severity of condition, e.g., cortical Lewy

pathology in advanced PD, and whether there are differences in the change from baseline in brain [GSH] between subject types. In this study we focused on the occipital cortex rather than the substantia nigra, where a GSH deficit has been detected postmortem in PD.[236]Due to challenges associated with MRS in the substantia nigra in humans, including its location, very small size (limiting signal-to- noise ratio), and high iron content (giving rise to broad intrinsic linewidths, thereby limiting spectral resolution),[237,238] we chose the occipital cortex to increase our chances of reliably detecting a NAC effect on [GSH]. Our results suggest that the effect of NAC on [GSH] is global and that NAC is able to increase brain [GSH] even in controls.

Following a single intravenous NAC dose, we observed a transient increase in blood GSH redox ratio which was attributed to an increase in GSH as well as a decrease in GSSG in all subjects. GSSG disassociation by NAC, resulting in a NAC-GS complex and free GSH, could contribute to the transient nature of this change. Despite a rapid decrease in whole blood GSH redox ratios following the end of the NAC infusion, [GSH] in the brain remained elevated at 2 hours after the start of infusion. It is unknown whether the pharmacologic actions of NAC will be the same for oral administration.

There are reports of positive effects of oral NAC in disorders with CNS involvement.[239,240] Future studies are necessary to determine if oral NAC affects brain [GSH] and if so, to establish optimal dosage regimens to attain sustained elevation of brain [GSH] with repeated dosing.

Chapter 5

Future Studies of N-acetylcysteine in Neurodegenerative Disorders

5.1 Studies of Oral N-acetylcysteine

Although our studies show an increase in total brain GSH after NAC administration and improved survival outcomes in children with metabolic disorders after HCT with adjunctive NAC treatment, i.v. administration is clearly not practical for long term treatment with NAC, particularly in the out-patient setting. Questions remain however, regarding the bioavailability and therapeutic effects of NAC given orally. It is known that oral NAC undergoes extensive first pass metabolism, but what is yet unknown is whether its conversion and therefore supply of cysteine for the biosynthesis of GSH is the most important means of NAC exerting its therapeutic effects.

Arfsten et al studied the distribution of radio-labeled NAC (^{14}C-NAC) and its impact on glutathione metabolism in Sprague-Dawley rats following single and multiple dosing with NAC (1200 mg/kg) by oral gavage.[241] They found that radioactivity associated with administration of ^{14}C-NAC distributed to most tissues examined within 1 hour of administration; peak radioactivity levels occurred within 1 hour to 4 hours and for a majority of the tissues examined, radioactivity remained elevated for up to 12 hours or more. Administration of a second dose of NAC 4 hours after the first increased liver, kidney, skin, thymus, spleen, eye, and serum radioactivity significantly beyond levels achieved following 1 dose. Administration of a third dose of NAC + ^{14}C-NAC 4 hours after the second dose did not significantly increase tissue radioactivity further except in the skin. Interestingly, GSH concentrations were increased 20% in the skin

and 50% in the liver after one dose of 1,200 mg/kg NAC whereas lung and kidney GSH were unaffected. Administration of a second and third dose of NAC at 4 hours and 8 hours after the first did not increase tissue GSH concentrations above background with the exception of skin GSH levels, which were elevated to levels similar to those obtained after a single dose of NAC.

Levels of glutathione-S-transferase (GST), the enzyme responsible for linking toxic compounds with GSH, were increased 150% in the kidney and 10% in the liver, decreased 60% in the skin, and had no effect on lung GST activity following a single dose of 1,200 mg/kg NAC. Administration of a second dose of NAC 4 hours after the first decreased skin GST activity a further 20% whereas kidney GST activity remained elevated at levels similar to those obtained after 1 dose of NAC. Administration of a third dose of NAC 4 hours after the second dose increased liver GST activity significantly as compared to background but did not affect skin, kidney, or lung GST activity.

Transient decreases in glutathione reductase (GR) activity were measured in the skin and kidney in association with repeat administration of 1,200 mg/kg NAC. Glutathione peroxidase (GxP) activity was increased in the skin, kidney, and liver, suggesting that oxidative stress was occurring in these tissues in response to repeat dosing with NAC.

Overall, the results of this study present the possibility that NAC could provide some benefit in preventing or reducing toxicity related to oxidative stress in some tissues by increasing tissue NAC and/or cysteine levels, GSH concentrations, and GST activity. The authors suggest that follow-on studies are necessary and that the effect of NAC on increases in GxP needs to be further investigated.

Future studies of orally administered NAC could use a stable-labelled, nonradioactive NAC to characterize the effects of NAC on intracellular cysteine and glutathione concentrations. Using this technique, one could compare the intracellular concentrations of NAC and is metabolic products cysteine and glutathione and compare the effects of i.v. versus oral administration to determine if oral administration produces the desired treatment effects. Since the safety of stable-labelled compounds have been established, these studies could be performed in either children with metabolic disorders or adult patients with Parkinson or Gaucher disease. If oral administration of NAC is found to have similar intracellular and brain effects as i.v. administered NAC, this would pave the way for larger scale studies of appropriate oral doses of NAC in patients with neurodegenerative and other diseases where glutathione is found to be deficient. In the case of ALD, if oral NAC is found to have positive effects on intracellular and brain GSH concentrations, oral administration of NAC long-term could be a viable option in treatment of newly diagnosed ALD to potentially prevent the onset of the cerebral form of the disease, for which there is no known cure at present, and it would present an option with a much lower risk than HCT

or gene therapy. For those with Parkinson disease or Gaucher disease, oral NAC therapy initiated early in the course of disease could represent a treatment option to delay or prevent the progression of the disease.

5.2 Studies of N-acetylcysteine amide

Despite decades of research on the effects of NAC in numerous and diverse disorders, it is still used routinely on a widespread basis only as an antidote for acetaminophen toxicity and as a mucolytic. In a recent critical review of NAC, the author concluded, "NAC has so far not fulfilled the impressive promises that theory and experimental research have put forward, thus it seems to share the destiny of other antioxidants"[242] Failure of NAC to provide significant effect may be due in part to its low oral bioavailability or its hydrophobicity. At physiological pH, NAC acquires a negative charge by losing a proton from the carboxyl group. This deprotonation hinders its passage across biological membranes.[243] In critical oxidative stress conditions, adequate concentrations of NAC may not be present to replenish the GSH concentrations. In addition, NAC has been found to be pro-oxidant at higher concentrations.[244] These limitations may in part explain the lack of success in the wide variety of disorders in which NAC has been tested.

In recent years, lipophilic thiol compounds with improved bioavailability and membrane permeability have been proposed. N-acetylcysteine amide (NACA) is one such compound. NACA is a derivative of NAC in which the carboxyl group is

neutralized by an amino group with the expectation of improving the lipophilicity and membrane permeability of the compound. A recent study provided evidence that NACA had more efficient membrane permeation than NAC and could replenish intracellular GSH in red blood cells, possibly by disulphide exchange with oxidized glutathione (GSSG).[245] NACA was also shown to cross the blood brain barrier, scavenge free-radicals, chelate copper, protect red blood cells from oxidative stress and attenuate myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis in a multiple sclerosis mouse model.[245] NACA is partially metabolized to N-acetylcysteine.[246]

5.2.1 NACA in Alzheimer's disease

GSH depletion and oxidative stress are believed to play a central role in the deterioration of neuronal tissue associated with aging and Alzheimer's disease (AD). It is well established that the protein amyloid-beta (Aβ) plays an important role in the pathogenesis of AD and has the ability to induce oxidative stress in brain tissue. Prevention of Aβ- induced oxidative stress in brain tissue may provide a potential treatment for AD. Bartov et al experimented with NACA in a rat model of AD to determine if NACA could protect neuronal cells from the toxic effects of Aβ.[247] Rat primary neurons were treated with 10μM NACA or saline one hour prior to the addition of 10μM Aβ. Cell viability, protein oxidation and lipid peroxidation were measured following a 24 hour incubation. Pretreatment with NACA was able to protect rat neuronal cells from the toxic effects of Aβ and prevent oxidative damage to neuronal proteins and lipids. NACA's ability to

protect neuronal cells from the toxic effects and oxidative stress of Aβ provide some support for the potential benefit of antioxidant treatment with NACA in AD. It should be noted that NAC has been studied in a clinical trial of patients with moderate to severe AD to determine if NAC treatment could improve the course of neurologic deterioration in AD.[226] In the double blind placebo-controlled clinical trial of oral NAC, 50mg/kg/day in three divided doses daily was given to moderately to severely affected patients. The primary endpoint was the score on the Mini Mental Status Exam (MMSE) and activities of daily living (ADLs). Secondary outcomes included various neuropsychological tests. Outcomes were compared at 12 and 24 weeks. The active (n=23) and placebo (n=20) groups were well matched at baseline. At mid-point evaluation, the analysis showed a trend toward a favorable treatment effect on MMSE score in favor of NAC (p=0.056). At 24 weeks, patients receiving NAC showed significantly better performance on the letter fluency task compared to placebo. Performance on the Wechsler Memory Scale immediate figure recall showed a trend toward improvement by NAC (p=0.067), but the trial failed to meet its primary endpoint. Several factors may have affected the results of this trial: 1.) the study was likely underpowered to see a treatment effect on MMSE, 2.) the inclusion criteria may have been too broad (a MMSE score of 12-26 will include mildly and severely impaired patients, and 3.) the dose of 50 mg/kg of NAC was likely not high enough to show an effect. It may be possible for a potent thiol antioxidant with better brain penetration to show an effect in a well-designed study of AD.

5.2.2 NACA in Parkinson disease

As discussed earlier, the symptoms of Parkinson disease are due, at least in part, to the loss of dopaminergic neurons in the substantia nigra. It is believed that oxidative stress plays an important role in dopaminergic neuron loss. Animal models for PD commonly utilize toxins (6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP) and rotenone) to selectively destroy dopaminergic neurons. These toxins are also known to produce oxidative stress. A study by Bahat-Stroomza et al investigated the neuroprotective effects of NACA in three animal models of PD: 1.) amphetamine-induced rotational behavior in unilateral 6-OHDA-induced nigral lesioned rats, 2.) MPTP treated mice, and 3.) rotenone treated rats.[248] In the 6-OHDA rat model, 120mg/kg NACA or saline was administered intraperitoneally (i.p.) 15 minutes before the 6-OHDA lesion. The rats were allowed to recover for 14 days and then amphetamine-induced rotations were counted for 2 hours following administration of amphetamine 5mg/kg i.p. The intensity of the amphetamine-induced rotation is directly proportional to the extent of the unilateral 6-OHDA nigral lesions.[249] In this rat model, NACA treatment markedly reduced amphetamine-induced rotations by 49% ($p<0.001$).

In the MPTP model, C57/bL mice were administered 25 mg/kg MPTP i.p. once daily for 5 consecutive days. During the 5-day treatment period, NACA 1g/kg/day was administered in drinking water. At the end of the 5-day treatment period striatal dopamine levels were measured. MPTP treatment reduced striatal

dopamine content by 51% compared with control mice ($p<0.05$). Administering NACA in drinking water during the 5-day treatment period with MPTP almost completely prevented the MPTP induced reduction in striatal dopamine content. In the rotenone model, rats were administered rotenone 5mg/kg/day for 28 days intravenously. During the 28-day treatment period, NACA, 100mg/kg/day was administered in drinking water. At the end of the treatment period nigral dopaminergic neurons were counted. Chronic (28-day) daily administration of rotenone markedly reduced dopaminergic neurons in these rats. However, administering NACA in the drinking water during the 28-day treatment period completely abolished the rotenone induced reduction in dopaminergic neurons. In this study, NACA demonstrated marked activity in three independent animal models of PD. These consistent results across animal models suggest that NACA may be beneficial for the treatment of PD.

5.2.3 NACA in Multiple Sclerosis

Multiple sclerosis (MS) is a chronic, progressive autoimmune disease targeting the white and gray matter of the brain and spinal cord. The autoimmune response causes inflammation, demyelination and nerve cell death. Currently there is no cure for MS; therefore treatment is focused on improving the quality of life and reducing the symptoms of the disease. Reactive oxygen species have been implicated as mediators of demyelination and nerve cell death in MS. It is known that oxidative stress activates certain transcription factors involved in MS, for example nuclear factor kappa B (NF-κB) which up-regulate the expression of

many cell factors involved in MS including tumor necrosis factor α (TNFα). Therefore, because of the role of oxidative stress in MS, it seems reasonable that antioxidants may provide a treatment option. In the model of experimental autoimmune encephalomyelitis (EAE), a well-accepted mouse model of MS, several studies have demonstrated the protective effects of antioxidants and suppression of EAE. Although a number of antioxidants have been studied clinically in neurodegenerative disorders, little benefit has been observed, presumably due to the limited penetration of these antioxidants into the CNS. Due to the proposed increased brain penetration of NACA, Offen et al studied NACA in the EAE mouse model of MS to characterize the ability of NACA to penetrate the brain, its antioxidant properties and its effects on motor function.[245]

NACA and NAC were administered intraperitoneally, 30mg/kg, and orally 160mg/kg to mice. Fifteen minutes after study drug administration the mice were sacrificed and NACA and NAC concentrations in the brain were determined. Following both i.p. and oral NACA administration, significant NACA concentrations could be detected in the mouse brains. In contrast, following oral and i.p. NAC administration, NAC could not be detected in the mouse brains, although an increase in cysteine was detected after NAC administration.

The ability of NACA and NAC to replenish GSH and prevent protein oxidation was also tested in human red blood cells (hRBCs). In GSH depleted hRBCs, both NACA and NAC increased the RBC thiol content (GSH + other thiols),

however NACA was more effective than NAC (220% versus 62% respectively at 5mM conc). The addition of tert-butylhydroxyperoxide (t-BHP) to hRBCs increased ROS and caused progressive loss of intracellular thiols and an increase in oxidized hemoglobin. In contrast, in hRBCs pretreated with NACA (5mM) prior to exposure to tBHT, intracellular thiol levels increased and normal levels of oxidized hemoglobin were observed, indicating the NACA protected the nRBCs from oxidative stress. Interestingly, a sharp decrease in intracellular thiols and an increase in oxidized hemoglobin were observed in hRBCs pre-treated with NAC (5mM) prior to tBHT exposure. The authors concluded that NACA is approximately threefold more efficient than NAC at restoring intracellular thiol pools, probably due to the better penetration into the cell. They also stated that "unlike NAC, NACA appeared to integrate into the redox machinery of the human RBC and effectively protect hemoglobin from oxidation."[244]

The EAE mouse model has many clinical and pathological similarities to human MS [250,251]; therefore this model was utilized to test NACA effects on motor function and development EAE. Initial *in vivo* experiments were performed to test the efficacy of NACA to suppress EAE when given orally in varying doses (5-50 mg/kg) twice daily. All mice that received only saline developed clinical signs while the NACA-treated mice showed a marked delay in the onset and a significant reduction in the severity of the clinical EAE signs. The next experiments explored the efficacy of NACA after the appearance of clinical signs.

EAE was induced in the mice by immunization on Day 1 and Day 8 with an emulsion containing myelin oligodendrocyte glycoproteins (MOG). Clinical symptoms of EAE developed within 14-18 days in the majority of animals. Mice were randomly assigned to one of four treatment groups: 1) control (daily saline administration), 2.) daily 200 mg/kg NACA i.p. from the first MOG injection, 3.) daily 200mg/kg NACA i.p.from Day 8 after the first MOG injection and 4.) daily 200mg/kg i.p. from day 14 after the first MOG injection (when the first EAE symptoms typically appear). At several time points following the first MOG injection, the mice were scored for EAE clinical signs: 0=no paralysis, 1=loss of tail tonicity, 2=mild hind limb weakness, 3=complete hind limb paralysis, 4=paralysis of four limbs, 5=total paralysis and 6=death.

In the EAE mouse model of MS, NACA administered from Day 1, Day 8, or Day 14 following the initial MOG injection was highly effective ($p<0.002$ compared to control) at reducing EAE clinical score. In addition, when NACA was administered after the appearance of symptoms on Day 14 following the initial MOG injection, a marked improvement ($p<0.002$ compared to control) in EAE clinical score was observed. Consistent with these reductions in clinical score, reduced inflammation and absence of inflammatory cells were observed in spinal cords from NACA-treated mice compared with control, saline-treated mice.

These studies indicate that NACA may have promise as an antioxidant in neurodegenerative disorders, and that it may be a more potent antioxidant, with

increased ability to penetrate the blood brain barrier and cell membranes than NAC, at concentrations that are non-toxic.

Chapter 6

Conclusion

Neurodegenerative disorders such as Alzheimer's disease, Parkinson disease and multiple sclerosis are common disorders affecting millions in the U.S. alone. While treatments are currently available, they are symptomatic treatments, as cures for these disorders remain elusive. Other neurodegenerative disorders such as adrenoleukodystrophy, Huntington's disease and amyotrophic lateral sclerosis, although less common, are devastating disorders with variably effective treatments and no cure. All of these disorders share a common element involved in their pathogenesis and/or progression: oxidative stress.

Oxidative stress is generated in neurons when the metabolic balance is overwhelmed and the sum of free radicals generated by oxygen metabolism is greater than the capacity of the cell to detoxify these substances. Oxidative stress is common in the brain, as it uses a disproportionate amount of oxygen per volume of tissue compared to other organs.[1] Glutathione (GSH) is the most prevalent endogenous antioxidant in the brain. It exerts its antioxidant effects non-enzymatically by reacting with superoxide[7], nitric oxide (NO) [8] hydroxyl radicals[9], and peroxynitrite ($ONOO^-$).[10] GSH also serves as an electron donor for the reduction of H^2O^2 or other peroxides catalyzed by GPx.[12] The ratio of reduced to oxidized glutathione (GSH/GSSG) serves as an indicator of the cellular redox environment.[14] GSH also serves as a non-toxic form of cysteine storage,[16] a major redox buffer,[17] and a neurotransmitter/neuromodulator.[18] Since GSH is the most important antioxidant in the brain, its depletion is an important factor in the pathogenesis of many neurodegenerative disorders;

whether GSH depletion is a cause or one of the effects of disease is still in question.

Since the search for a cure for most neurodegenerative disorders is ongoing, trials of antioxidants that could slow the progression or perhaps ultimately prevent these disorders is a worthwhile endeavor. Several antioxidants, including vitamin C and E, Co-enzyme Q-10, and even glutathione itself have been studied in Parkinson's, Alzheimer's and Huntington's disease,

MS and other neurologic disorders without much success, due to their inability to effectively cross cellular membranes and the blood brain barrier. N-acetylcysteine (NAC) is a thiol antioxidant that has been tested in the clinical setting for a variety of disorders characterized by oxidative stress, due to its efficiency in synthesis and replenishment of GSH in addition to its ability to scavenge reactive oxygen species (ROS). Besides antioxidant activity, NAC exerts numerous key pharmacological actions. NAC reduces disulfide bonds that crosslink glycoproteins in mucus and thus acts as mucolytic agent. It exhibits anti-inflammatory properties by inhibiting NF- κ B activation. It is also a chelator of heavy metals including mercury, lead, and arsenic. It acts as a precursor of glutamate which plays a major role in the neurobiology of mood, psychosis, and addiction. Further, NAC has been considered as cardioprotectant as it decreases the elevated levels of homocysteine. Furthermore, NAC has also been shown to

play a major role in vasodilation, neutrophil activation, and microbial attachment, and interferes with redox signaling.[252,253]

The studies reported in this thesis include a pharmacokinetic study of NAC in young boys with X-linked adrenoleukodystrophy undergoing hematopoietic stem cell transplant, and a pharmacokinetic/pharmacodynamics study of NAC in adult patients with Parkinson's disease and Gaucher disease. In the first study, my research colleagues and I report for the first time, the pharmacokinetic parameters of NAC in pediatric patients and assess the effect of HSCT and the pre and post- transplant immunosuppressants on NAC PK parameters. In the second study we report, also for the first time, the direct effects of NAC administered intravenously on brain glutathione concentrations. Both of these preliminary studies add to our knowledge of the pharmacokinetics of NAC and its effects on brain GSH concentrations and should assist with design of larger, placebo-controlled trials of NAC in neurodegenerative disorders. What is yet to be determined in a well-designed trial, is whether orally administered NAC would have similar effects on brain GSH.

Although NAC has become a researcher's preferred antioxidant to treat various pathologies associated with oxidative stress, and has shown beneficial effects in experimental animals and some clinical trials, few clinical trials have shown a robust effect of NAC and it is still only used routinely in acetaminophen overdose and as a mucolytic agent. The failure of NAC to show significant effect may be

due to its low bioavailability and hydrophobicity. At physiological pH, NAC acquires a negative charge by losing a proton from the carboxyl group. This deprotonation hinders its passage across the biological membrane.[243] In a critical oxidative stress condition, the concentration of NAC may not be enough to replenish the intracellular GSH content. Moreover, a few studies have demonstrated the pro-oxidant nature of NAC at higher concentrations.[244]

In spite of these limitations, the use of thiol antioxidants in clinical settings is warranted to counteract the excess ROS. Following systemic delivery, the drug has to reach the target site in a steady-state concentration. In this regard, antioxidant compounds with better membrane permeability may become vital molecules to target the site of excess ROS production, especially the brain. One such potential molecule is N-acetylcysteine amide (NACA), a derivative of NAC in which the carboxyl group is neutralized by an amino group. NACA has been shown to cross the blood-brain barrier as well as other biological membranes more effectively than NAC. Additionally, NACA has been shown to be effective in several animal models of neurodegenerative disease, including models of Parkinson's disease and multiple sclerosis.

The research included in this thesis lays the groundwork for future studies of NAC and its derivative NACA in the potential treatment of neurodegenerative disorders which include critical levels of oxidative stress and glutathione depletion. Until such time as a cure is discovered, hopefully compounds such as

these can bring symptom relief and hope to those who suffer from the devastating effects of disorders such as Parkinson disease, Gaucher disease, and particularly adrenoleukodystrophy.

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